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14. ABSTRACT The transcription factor NF-κB controls several aspects of cancer including initiation, progression, metastasis and resistance to chemotherapy. We had previously shown repression of the pro-apoptotic gene GADD153 by NF-κB in breast cancer cells. GADD153 is induced when cells are exposed to environmental stress/toxicants or deprived of nutrients. GADD153 induces apoptosis of cells with damaged DNA, which may be an essential strategy for cancer prevention. By inhibiting GADD153, NF-κB may allow accumulation of cells with damaged DNA and such cells are more prone for immortalization and transformation. In this study, we have identified NF-κB repressible element in GADD153 promoter, which binds to Snail/Gfi family of transcription repressors. Thus, NF-κB may repress gene expression by upregulating the expression of Snail/Gfi family transcription repressors. We used the immortalized mammary epithelial cell line MCF10A to determine the consequences of constitutive NFκB activation. MCF10A cells overexpressing the p65 subunit of NF-κB showed fibroblastic phenotype, which resembled the phenotype of epithelial cells that have converted to mesenchymal type (Epithelial to mesenchymal transition, EMT). EMT phenotype of p65 overexpressing cells correlated with reduced expression of epithelial specific markers E-cadherin, Desmoplakin and Keratin 18, and increased expression of mesenchymal markers Fibronectin and Vimentin compared to parental cells. MCF10A cells overexpressing p65 showed elevated expression of transcription repressors ZEB-1 and ZEB-2. Overexpression of ZEB-1 alone was sufficient for EMT of MCF10A cells. Chronic exposure of MCF10A cells to tumor necrosis factor, a potent inducer of NF-κB, also resulted in EMT. P65 overexpression in MCF10A variants with myoepithelial phenotype, based on p63 expression pattern, resulted in loss of p63 expression. Myoepithelial cells are usually converted to myofibroblasts during invasive phase of breast cancer. Based on these observations, we propose that NF-κB promotes initiation and progression of breast cancer by modulating the expression of GADD153, possibly through Snail/Gfi family transcription factors, and by inducing EMT through ZEB-1 and ZEB-2.					
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## INTRODUCTION:

Understanding the mechanism of breast cancer initiation is critical for developing chemoprevention strategies. Normal mammary epithelial cells from which cancer usually originate have limited life span. Immortalization is the first step that leads to continuous growth of mammary epithelial cells (1). Cell cycle regulatory, anti-apoptotic and pro-apoptotic proteins play a significant role in immortalization process (1). In addition, recent studies suggest that oncogene activation leads to senescence, which serves as an initial barrier for transformation and senescence associated genes are silenced during immortalization (2-5). Immortalized cells attain cancerous growth properties (transformation) due to additional mutations that lead to either loss of tumor suppressor genes or activation of oncogenes. The transcription factor Nuclear factor-kappaB (NF- $\kappa$ B) promotes both immortalization and transformation by controlling the expression of cell cycle regulatory, anti-apoptotic and pro-apoptotic genes (6). NF- $\kappa$ B is usually sequestered in the cytoplasm of resting cells through its association with inhibitor-of-kappaB (I $\kappa$ B) proteins and translocates to nucleus upon exposure of cells to cytokines and growth factors (7). NF- $\kappa$ B then binds to response elements and induces the expression of genes involved in invasion, metastasis and chemotherapy resistance. We and others have shown that NF- $\kappa$ B is constitutively active in breast cancer and is responsible for overexpression of several anti-apoptotic genes as well as repression of the pro-apoptotic growth arrest and DNA damage/C/EBP homology protein (GADD153/CHOP) (8-11). GADD153 is induced when DNA is damaged or cells are under stress. Depending on the extent of damage, cells either repair DNA damage and survive or die. GADD153 is believed to promote death of cells with severe damage, thereby limiting accumulation of cells with mutations (12). Thus, GADD153 is likely to play a role in preventing breast cancer initiation. Because NF- $\kappa$ B reduces GADD153 expression, it is possible that cells that contain constitutively active NF- $\kappa$ B will survive after DNA damage and these cells are more prone for transformation. This award was to test this possibility. Three aims were proposed: 1) To determine whether inhibition of GADD153 by NF- $\kappa$ B is essential for survival and/or transformation of MCF-10A cells when exposed to MMS or grown under nutrient-deprived condition. 2) To determine whether inhibition of GADD153 by NF- $\kappa$ B leads to altered activity of the transcription factor C/EBP $\beta$  and differentiation of MCF10A cells. 3) To determine the influence of p53 on the anti-apoptotic function of NF- $\kappa$ B in MCF-10A cells grown under nutrient-deprived condition or exposed to MMS.

## BODY:

### Specific Aim I:

Task 1: (months 1-5) Establish MCF10A cells overexpressing p65NLS50 or ras and characterize them with respect to constitutive NF- $\kappa$ B activity.

Results: This task is completed. We have established MCF10A cells overexpressing p65NLS50 or Ras (Figure 1A and B). MCF10ApQ and MCF10Aneo cells correspond to parental cells vector alone. MCF10A cells overexpressing p65 (MCF10A/p65 series 1 to 4) showed elevated levels of p65 as well as NF- $\kappa$ B-inducible genes including cyclin D1, Mn-SOD and I $\kappa$ B $\alpha$  (Figure 1A).



Task 2: (Months 6-8) Determine MMS-inducible and nutrient-deprivation inducible expression of GADD153 in MCF10/p65NLS50 cells by Northern and Western blots. Determine apoptosis by Annexin V labeling, PARP cleavage and DNA laddering.

Results: Cell viability experiments were carried out using the MTT assay and clonogenic assays. MCF-10A/p65NLS50 cells treated with MMS (0 to 10 mM) for 3 to 24 hrs did not show a significant difference in cell viability, as measured by MTT assay compared to vector control cells (data not shown). However, while parental cells (MCF10A/pQ1) were growth inhibited by transforming growth factor beta, MCF-10A/p65(1) cells were resistant (Figure 2A). Unlike in cancer cells, serum starvation did not induce GADD153 in MCF10A cells. Interestingly, MMS-inducible expression of GADD153 was delayed in MCF10A/p65 cells compared to MCF10A/pQ cells (Figure 2B). Thus, NF- $\kappa$ B activation protects mammary epithelial cells against only specific agents.

Task 3: Months (9-15) determine the susceptibility of MCF10A, ras and p65NLS50 cells to MMS-induced transformation. This will be achieved by growing cells in soft agar and matrigel.

Results: MCF10ApQ cells plated on a Matrigel form a highly organized acinar structure. These cells have been used extensively as a model system to study the formation and maintenance of glandular architecture *in vitro*. These acinar structures consist of a hollow lumen surrounded by a single layer of cells, with the hollow lumen formed through a selective apoptosis of centrally located cells (13). Oncogenes such as ErbB2 prevent apoptosis and induce proliferation of the centrally located cells, which leads to the formation of a highly disorganized structure (14). As expected, the parental MCF10A/pQ cells cultured in matrigel formed polarized acinar structures (Figure 3). In contrast, MCF10A/p65 cells formed disorganized structures. Thus, NF- $\kappa$ B activation alone is sufficient for MCF10A cells to show a transformed cell phenotype in matrigel.

*Deregulated growth of MCF10Ap65 cells correlates EMT phenotype.* When grown in two-dimensional culture, MCF10A/p65 cells exhibited a fibroblastic, spindle-shaped morphology, whereas MCF10A/pQ control cells retained an epithelial-like morphology (Figure 4A). The morphological changes observed in MCF10A/p65 cells resembled that of cells undergoing EMT. To test this possibility, northern and western analyses of epithelial and mesenchymal markers were carried out. MCF10A/p65 cells showed reduced expression levels of epithelial markers, including E-cadherin, Occludin, Desmoplakin and Keratin 18 (K18) compared to MCF10A/pQ control cells. In contrast, expression of the mesenchymal markers Vimentin, and Fibronectin were increased in MCF10A/p65 cells compared to parental cells (Figure 4B).

*Increased cellular motility of MCF10A/p65 cells.* Increased motility is another hallmark of EMT. Using the *in vitro* wound healing assay, we observed that MCF10A/p65 cells showed a faster rate of motility compared to MCF10A/pQ control cells when monitored over a period of 24 hrs (Figure 5). Taken together, these results confirm NF- $\kappa$ B-dependent EMT of MCF10A cells.



*Induction of ZEB-1 and ZEB-2 expression by NF- $\kappa$ B.* To identify downstream targets of NF- $\kappa$ B involved in induction of EMT phenotype, we used Northern blot and RT-PCR to analyze the expression of transcriptional regulators involved in EMT. MCF10A/p65 cells showed increased expression of the transcriptional repressors ZEB-1 and ZEB-2 (Figure 6A), but not of other transcriptional repressors Snail-1, -2 or -3 (Figure 6B). These results are distinct from that observed in *Drosophila*, where the NF- $\kappa$ B homologue dorsal induces EMT through Snail (15). We did not observe a difference in the expression levels of CtBP-1 and CtBP-2 (data not shown), both of which are co-factors required for ZEB-1 and ZEB-2 mediated transcriptional repression (16). IL1- $\alpha$  or TNF $\alpha$ , which induce NF- $\kappa$ B, increased ZEB-1 and ZEB-2 expression in MCF10A and MDA-MB-231 cells, respectively (Figure 6C).

*Overexpression of ZEB-1 induces EMT phenotype in MCF10A cells.* To determine whether ZEB-1 alone can induce EMT, MCF10A cells were transduced with control retrovirus (pQCXIN) or with ZEB-1/pQCXIN retrovirus encoding ZEB-1. Two independent clones of MCF10A cells overexpressing ZEB-1 were phenotypically similar to MCF10A/p65 cells (Figure 7A). Northern blot analysis showed higher ZEB-1 and Vimentin, but lower E-cadherin and Desmoplakin levels in MCF10A/ZEB-1 cells compared to control MCF10A/pQN cells. Northern blot analysis was carried out in triplicate for statistical analysis (Figure 7B). These results suggest that ZEB-1 alone can induce the EMT phenotype in MCF10A cells.

*Induction of EMT phenotype in MCF10A cells through chronic exposure to TNF $\alpha$ .* The experiments described so far demonstrating induction of the EMT phenotype by NF- $\kappa$ B were carried out in an artificial system involving p65 overexpression. To determine whether NF- $\kappa$ B when activated under more physiological conditions could induce the EMT phenotype, MCF10A cells were continuously cultured in medium containing TNF $\alpha$ , a known activator of NF- $\kappa$ B. TNF $\alpha$  did not cause any apparent cytotoxicity to the MCF10A cells and after 22 days of culture, the MCF10A cells showed induction of the EMT phenotype. TNF $\alpha$ -treated cells exhibited a fibroblastic morphology in two-dimensional culture and formed disorganized structures in three-dimensional Matrigel culture, a phenotype reminiscent of that observed with MCF10A/p65 cells (Figure 8).

*Reversal of EMT phenotype following removal of TNF $\alpha$  treatment.* To determine if the TNF $\alpha$ -induced EMT is reversible, cells treated with TNF $\alpha$  for 22 days were cultured in fresh media without TNF $\alpha$ . Interestingly, the MCF10A cells showed a gradual reversal of the EMT phenotype beginning from 11 days after TNF $\alpha$  removal and acquisition of epithelial morphology by day 18 of TNF $\alpha$  removal (Figure 9A). Reversal of EMT phenotype correlated with corresponding decrease in ZEB-1 and Vimentin expression and increase in E-cadherin and Desmoplakin expression (Figure 9B). Overall, the above experiments involving chronic TNF- $\alpha$  treatment suggest that NF- $\kappa$ B activation is essential for both initiation and maintenance of EMT phenotype.

## **Specific aim II:**



Task 1: Determine the DNA binding pattern of C/EBP $\beta$  and C/EBP $\beta$ :GADD153 heterodimers in various cell types by EMSA.

Results: Three isoforms of C/EBP $\beta$  are expressed in mammary gland. Normal mammary gland expresses full-length C/EBP $\beta$ -1 whereas breast cancers express shorter C/EBP $\beta$ -2 (17). C/EBP $\beta$ -1 and C/EBP $\beta$ -2 are derived from the same transcript utilizing different in-frame initiation codons and differ only by 24 amino acids. Overexpression of C/EBP $\beta$ -2 alone is sufficient for EMT of MCF-10A cells (18). We did not observe any difference in the mRNA levels of C/EBP $\beta$  in MCF10A/pQ and MCF10A/p65 cells (Figure 10A). However, we observed a switch in C/EBP $\beta$  isoforms at protein level upon overexpression of p65. While the MCF10A/pQ cells expressed ~50 kDa protein, which corresponds to C/EBP $\beta$ -1 isoform, MCF10A/p65 cells expressed C/EBP $\beta$  protein of ~46 kDa (Figure 10B). This is most likely C/EBP $\beta$ -2 isoform. Overexpression of C/EBP $\beta$ -2 isoform has been shown to increase cyclin D (18), which was also observed in MCF10A/p65 cells compared to MCF10A/pQ cells (Figure 10B).

Task 2: Determine the expression levels of C/EBP $\beta$  and GADD153-responsive genes in various cell types by transient transfection, Northern and Western blots.

Results: We have measured expression level of  $\beta$ -casein in MCF-10A/pQ and MCF10A/p65 cells. Consistent with changes in C/EBP $\beta$  protein expression pattern, we observed increased expression of cyclin D1 and osteopontin, C/EBP $\beta$  responsive genes (Figure 10A and B). Surprisingly, in EMSA, binding of proteins to C/EBP homodimers binding elements as well as to C/EBP/CHOP heterodimer binding elements was reduced in MCF10A/p65 cells compared to parental MCF10A/pQ cells (Figure 10C).

Task 3: Months: Determine the differentiation pathway in various cell types in response to lactogenic stimulation. This will be achieved by measuring  $\beta$ -casein and WAP expression in response to prolactin treatment.

Results: Without any prolactin treatment, we have observed significant change in differentiation program in MCF-10A/p65 cells as outlined below.

In initial studies, MCF10A cells were described as ductal luminal epithelial cells whereas a recent study described MCF10A as myoepithelial cells (19, 20). One explanation for the controversial observation is that MCF10A cells correspond to breast stem cells, which can differentiate into myoepithelial and ductal epithelial cells based on growth condition. Cytokeratin 5 (CK5) is considered as a marker for progenitor/stem cells that can differentiate to either glandular cells (CK8/18+) or myoepithelial cells (SMA+) cells (21). P63, a p53 homologue, is considered as a marker for myoepithelial cells (22). P63 is expressed as several different isoforms; TAp63 and  $\Delta$ Np63 are the major isoforms derived from alternative promoters. While TAp63 has a transactivation domain and can activate gene expression through p53 response element,  $\Delta$ Np63 can dominantly inhibit p53 function.  $\Delta$ Np63 is most commonly expressed isoform in myoepithelial cells (23).

Early passage MCF10A cells (Passage number ~180) obtained from Michigan Cancer Foundation and a late passage cells obtained from our collaborator at our institution were first examined for CK5/6 expression by Western blotting. CK5/6



expression was observed in cells from both sources although expression was substantially lower in early passage cells compared to late passage cells (Figure 11A). In contrast, p63 expression was observed only in late passage cells (Figure 11A). Thus, the late passage cells have acquired some of the characteristics of myoepithelial cells. Most of the studies described below were done using late passage cells because myoepithelial cells have been shown to be replaced by cells that have converted from epithelial to mesenchymal phenotype when breast cancer progresses to invasive phenotype (24).

Because MCF10A/p65 cells showed fibroblastic phenotype compared to MCF10A/pQ cells, we examined the effect of p65 on p63 expression by Western blotting. The p63 expression was observed in MCF-10A/pQ cells but not MCF-10A/p65 cells (Figure 11C), which suggests that NF- $\kappa$ B converts myoepithelial cells to myofibroblasts. Northern analysis for p63 further confirmed the results of Western analysis (Figure 11B and C)). MCF-10A cells exposed to TNF, IL-1 and TPA showed reduced levels of  $\Delta$ Np63, which suggests NF- $\kappa$ B-dependent repression of  $\Delta$ Np63 (Figure 11D). ZEB-1 may mediate NF- $\kappa$ B-mediated repression of p63 because p63 levels were lower in MCF-10A cells overexpressing ZEB-1 compared to parental cells (Figure 11E). These results clearly suggest that NF- $\kappa$ B can alter differentiation of myoepithelial cells without the requirement of any costimulus.

### **Specific aim III**

Task 1: Establish cells overexpressing p53 dominant-negative mutants and characterize them for p53 activity. Stable cell lines overexpressing p53V144A will be established.

Results: We generated MCF-10A cells overexpressing dominant negative p53 (Figure 12B). Unfortunately, this particular variant of p53 failed to have a dominant negative affect on wild type p53 of MCF10A cells because doxorubicin-inducible activation of p53-responsive gene p21 was same in parental and dominant negative p53 overexpressing cells (Figure 12A). Therefore, we used retrovirus vectors with shRNA against p53 from Open Biosystems to generate MCF-10A cells lacking p53. However, this approach also proved not useful in generating MCF10A cells with reduced p53 (Figure 12B). We next examined whether p53 expression can be reduced by transient expression of p53 siRNA. Although p53 siRNA reduced p53 protein levels (Figure 12B), transfection reagent itself proved toxic to cells and appears to activate p53 because we observed higher levels of p53 in cells transfected with irrelevant luciferase siRNA.

Task 2: Determine MMS sensitivity, NF- $\kappa$ B activity and GADD153 expression in various cell types.

Result: This task could not be completed because of our inability to develop MCF10A cells lacking p53 activity

Task 3: Determine the MMS-induced transformation frequency of cells that express p53 dominant negative mutant.

Results: This task could not be completed because of our inability to develop MCF10A cells lacking p53.



**Mechanism of GADD153 repression by NF- $\kappa$ B:** We have performed several mechanistic studies to understand repression of GADD153 by p65:

a) Exon1 of GADD153 contains NF- $\kappa$ B repressible element: Analysis of a series of deletion mutants of GADD153 promoter-CAT reporter revealed the presence of NF- $\kappa$ B-repressible element in exon 1 of GADD153 gene (Figure 13A). The activity of the mutant lacking exon 1 (pG3mut1/CAT) was not repressed by p65 compared to wild type reporter (pG3a/CAT). Cloning of Exon 1 upstream of the GADD153 promoter (pa91pG3mut1/CAT) or downstream of the CAT reporter (pb pG3mut1/CAT) restored p65-mediated inhibition of CAT activity (Figure 13B). Exon 1 contains a transferable p65 repressible element as an RSV promoter/CAT reporter with exon 1 of GADD153 but not unrelated 91 base long sequence was repressed by p65 (Figure 13A). Further deletion analysis of Exon 1 revealed that +41 to +71 bp region of Exon 1 contains p65-repressible element (Figure 13C).

b) Transcriptional activity of p65 is required for repression of GADD153: A mutant of p65 with has reduced transactivation potential due to mutation of protein kinase A phosphorylation site (S276A) was less efficient in repressing GADD153 expression compared to wild type p65 (Figure 14). These results suggest that a protein whose expression is induced by p65 is responsible for the repression of GADD153 expression.

c) Gfi family members are involved in repression of GADD153: The 30 bp region in exon 1 of GADD153 contains a putative binding site for the Gfi repressor family. The members in this family include Snail, Slug, Gfi-1, Gfi-1B, IA-1 and Mlt1(25, 26). Most of these proteins function as transcription repressors. Electrophoretic mobility shift assay (EMSA) showed specific binding of Snail-1 and Gfi-1 to the 91 bp region exon 1 of GADD153 (Figure 15A and B). We also observed binding of Gfi-1 to GADD153 promoter in MDA-MB-231 cells by chromatin immunoprecipitation assay (Figure 16). In transient transfection assays, Gfi-1 repressed GADD153 expression (Figure 17). Snail is a NF- $\kappa$ B inducible gene (27), although we did not observe NF- $\kappa$ B-dependent induction of Snail in both MCF10A and MDA-MB-231 cells (data not shown). Transcription factor binding motif search revealed the presence of 1, 6 and 2 putative NF- $\kappa$ B binding sites in Gfi-1, Mlt1 and IA-1 genes, respectively. Although our initial studies suggested that Gfi1 is NF- $\kappa$ B-inducible, we could not reproduce this results in several other cell types. We are currently investigating whether Mlt1 and IA-1 are induced by NF- $\kappa$ B, which play a role in repression of GADD153.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- MCF10A subtypes with myoepithelial characteristics overexpressing p65 subunit of NF- $\kappa$ B show an EMT phenotype. EMT phenotype correlated with loss of expression of myoepithelial cell marker  $\Delta$ Np63.
- EMT correlates with NF- $\kappa$ B-dependent expression of EMT-associated transcription repressors ZEB-1 and ZEB-2. ZEB-1 alone could induce EMT.
- Chronic exposure of MCF10A cells to TNF $\alpha$ , an inducer of NF- $\kappa$ B, also induced EMT phenotype.



- MCF10A cells overexpressing p65 show altered expression pattern of C/EBP $\beta$  isoforms.
- The exon 1 of GADD153 contains NF- $\kappa$ B repressible element. NF- $\kappa$ B repressible element binds to Snail/Gfi family transcription repressors. Snail/Gfi family transcription repressors may play a role in NF- $\kappa$ B-mediated repression of GADD153.

**Reportable outcomes:** Three abstracts have been published and two manuscripts will be submitted in one month. Part of the data is also reported in a review written by the PI.

**Review article:** NF- $\kappa$ B and breast cancer. *Current Problems in Cancer* 26:277-310 (2002)

#### **Abstracts:**

1) Hui Lin Chua, Sunil Badve and **Harikrishna Nakshatri**. NF- $\kappa$ B induces epithelial to mesenchymal transition phenotype to immortalized mammary epithelial cells. NF- $\kappa$ B: from bench to bedside. Keystone symposia, Snow Bird, Utah (January 10-15, 2004).

2) An NF- $\kappa$ B repressible element in the 5' untranslated region of the pro-apoptotic GADD153/CHOP gene. Hui Lin Chua and Harikrishna Nakshatri. American Association for Cancer Research 94<sup>th</sup> Annual Meeting Washington, DC, June 10-14, 2003.

3) Hui Lin Chua, Sunil Badve and Harikrishna Nakshatri. NF-kappaB promotes epithelial-to-mesenchymal transition of immortalized mammary epithelial cells through ZEB-1 and ZEB-2. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11, 2005.

**Conclusions:** NF- $\kappa$ B can influence breast cancer progression by multiple mechanisms. This study showed a role for NF- $\kappa$ B in converting myoepithelial cells to myofibroblasts. Myofibroblasts constitute important stromal cells that provide secretory factors required for invasion and metastasis of breast cancer (28). We have also identified a nexus between NF- $\kappa$ B and Snail/Gfi family of transcription factors. While transcription activation by NF- $\kappa$ B is very well studied, transcription repression by NF- $\kappa$ B is not known. Because Snail/Gfi family members are transcription repressors, they may mediate transcription repression by NF- $\kappa$ B.

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Figure 1: A) Generation of MCF10A cells overexpressing p65NLS50. MCF10A cells were infected with control retrovirus (pQ series) or p65NLS coding retrovirus (p65 1-4). Four independent mass cultures after selection with G418 were analyzed for expression of p65 and NF- $\kappa$ B inducible genes by Western blotting. B) Ras expression in parental (MCF10A/neo) cells and ras overexpressing cells (MCF10A/ras). Lysate from a mammary tumor developed in a MMTV-Ras transgenic mice (MMT-11ras) was used as a positive control.

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Figure 10: A) P65 overexpression does not affect the level of C/EBP $\beta$  mRNA but increases osteopontin expression. C/EBP $\beta$  and osteopontin expression were measured by Northern analysis. MCF10A/pQ(A) cells correspond to control cells whereas MCF10A/p65 (A, B, J) correspond to cells overexpressing p65. B) MCF10A/p65 cells contain a unique form of C/EBP $\beta$  protein compared to MCF10A/pQ cells as measured by Western blotting.

Figure 10C: Transcription factor binding to an oligonucleotide with consensus binding sequence for C/EBP homodimer (C/EBP-C/EBP) or C/EBP/GADD153 heterodimer (C/EBP-GADD153) was determined by electrophoretic mobility shift assays using nuclear extracts from MCF10A/pQ and MCF10A/p65 cells. Oligonucleotide with a binding site for SP-1 was used as a control. Note the effect of p65 overexpression on transcription factor binding to C/EBP-C/EBP and C/EBP-GADD153 elements.

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Figure 12: A) Generation of MCF10A cells overexpressing dominant negative mutant of p53 (p53dnV143A). However, dominant negative mutant had no effect on doxorubicin-inducible expression of p53 target gene p21. Expression of p53 and p21 was determined by Western blotting. B) p53 levels are reduced in MCF10A treated transiently with siRNA against p53 but not in cells stably expressing shRNA (00546) against p53. P53 expression was measured by Western blotting.

Figure 13A: Exon 1 of GADD153 promoter contains p65 repressible element. Cos-1 cells were transfected with indicated CAT reporters (5 micrograms), p65 or p50 expression vectors (0.5 micrograms) and 2 micrograms of  $\beta$ -galactosidase expression vector pCH110. CAT activity was measured 48 hour after transfection. The pG3a/CAT correspond to wild type GADD153 promoter with sequences -247 to +91 (which includes exon 1). The pG3mut1/CAT contains -247 to +1 sequence of GADD153 promoter. Wild type RSV/CAT and RSV/CAT with exon 1 of GADD 153 (p91/CAT) or irrelevant 91 bases (pcontr91/CAT) is also shown. Note that p65 represses pG3a/CAT but not pG3mut1/CAT.

Figure 13B: Insertion of exon 1 upstream of -247 to +1 of GADD153 sequence (pa91 G3mut/CAT) or downstream of CAT gene (pb91 pG3mut1/CAT) in pG3mut1/CAT restores inhibitory effect of p65.

Figure 13C: pG3a/CAT reporter lacking sequences from +42 to +71 of GADD 153 (pG3mutb/CAT) was not repressed by p65 whereas pG3a/CAT reporter with +1 to +71 (pG3muta/CAT) was repressed by p65.

Figure 14: The p65 with mutation of S276 to alanine [p65( S276mut)] was less efficient than wild type p65 in repressing GADD153 promoter. P5W1/CAT corresponds to CAT reporter with -954 to +91 bases of GADD153 promoter. Note that p65S276mut is less efficient than wild type p65 in activating NF $\kappa$ B/CAT reporter.

Figure 15: A) The exon 1 of GADD153 binds to Snail-1. Cos-1 cells were transfected with control plasmid pCMV2 or Flag-epitope tagged Snail-1. Radiolabeled 91 bp-untranslated region of exon 1 of GADD153 was incubated with nuclear extract from transfected cells and DNA: protein complex was resolved on an acrylamide gel. A specific DNA: protein complex was observed with nuclear extract from Snail-1 transfected cells. Flag antibody disrupted this complex. B) Binding of Gfi-1 to exon 1 of GADD153. Experiments were done as in A. Gfi-1:DNA complex was detectable with nuclear extracts from cells transfected with Gfi-1. This complex was disrupted by Gfi-1 specific but not isotype antibody. Also Gfi-1 binding was reduced substantially when exon-1 with Gfi-1 binding site mutation was used as a probe (p91mutb).



Figure 16: Binding of endogenous Gfi-1 to GADD153 promoter was determined by chromatin immunoprecipitation assay. MD435 cells were used because these cells express higher levels of Gfi-1. Gfi-1 immunoprecipitates from cross-linked cells contain sequences corresponding to exon 1 but exon 4 of GADD153. Gfi-1 binding was higher in cells that are overexpressing Gfi-1 (MD435/Gfi-1) compared to cells transfected with vector (MD435/pQN). PCR amplification of input DNA is also shown.

Figure 17: Gfi-1 represses GADD153 promoter. Cos-1 cells were transfected with pG3/CAT or pG3/CAT with mutation of Gfi-1 binding site (pG3mutb/CAT) and p65 or Gfi-1 expression vector. PCH110 was used as an internal control to measure transfection efficiency. pG3a/CAT but not pG3mutb/CAT activity was lower in cells transfected with p65 or Gfi-1. Thus, Gfi-1 functions as repressor of GADD153.



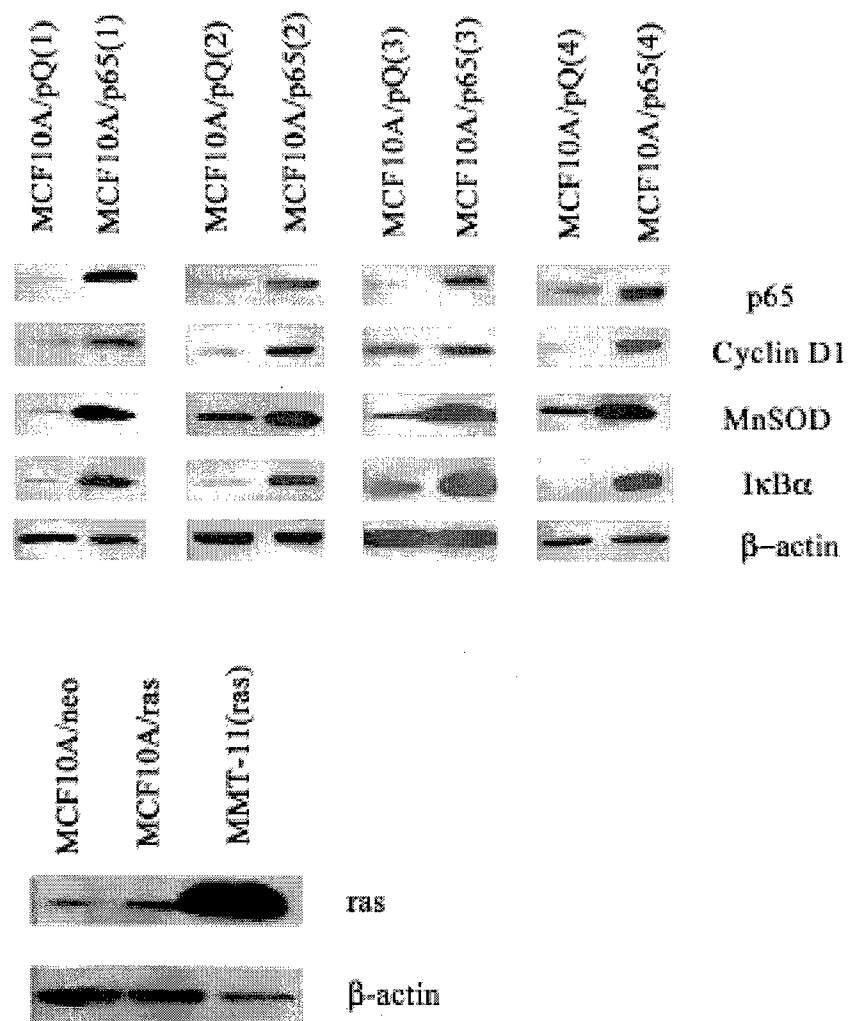


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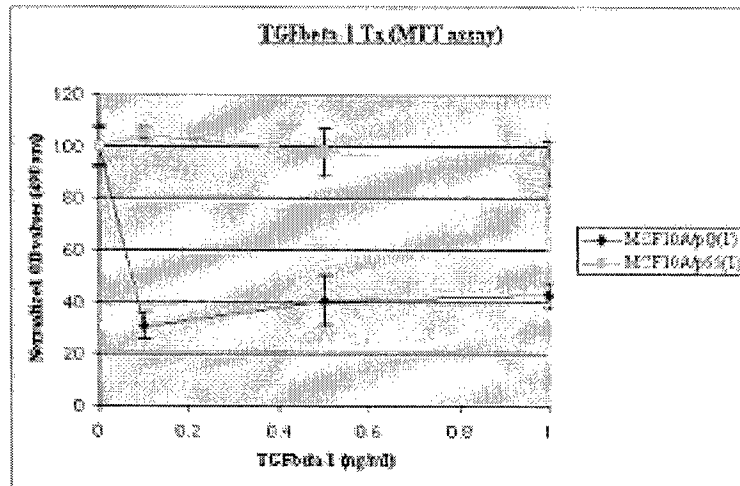
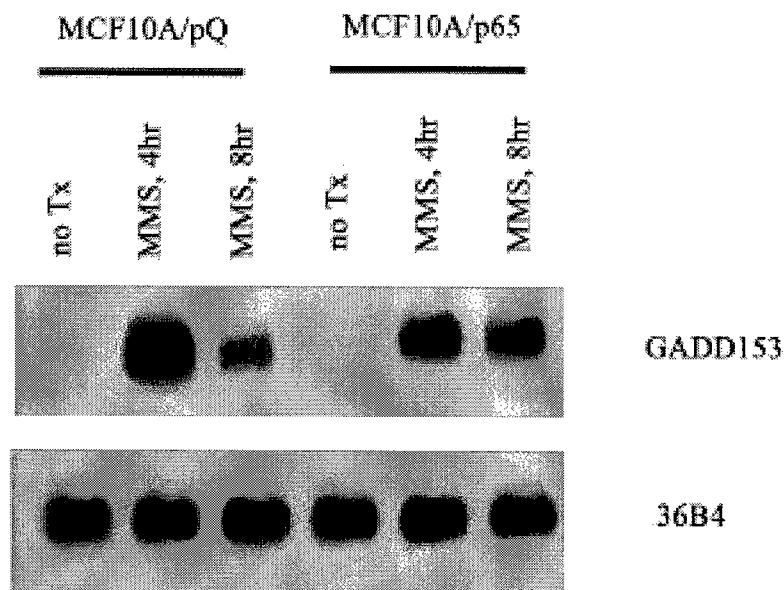
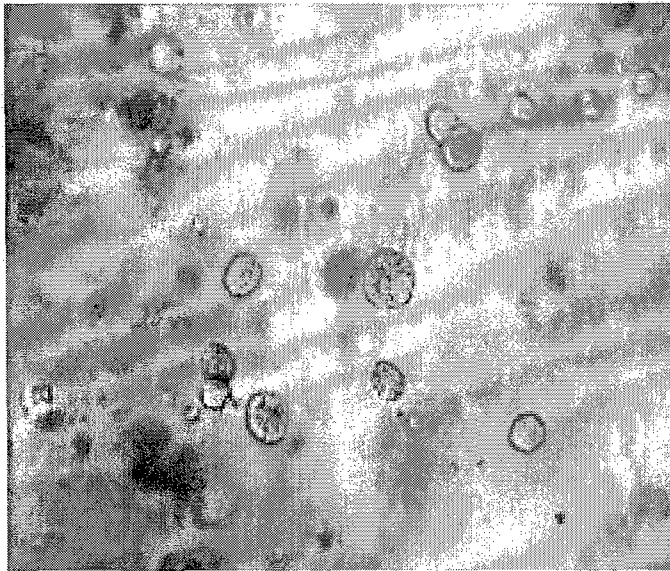
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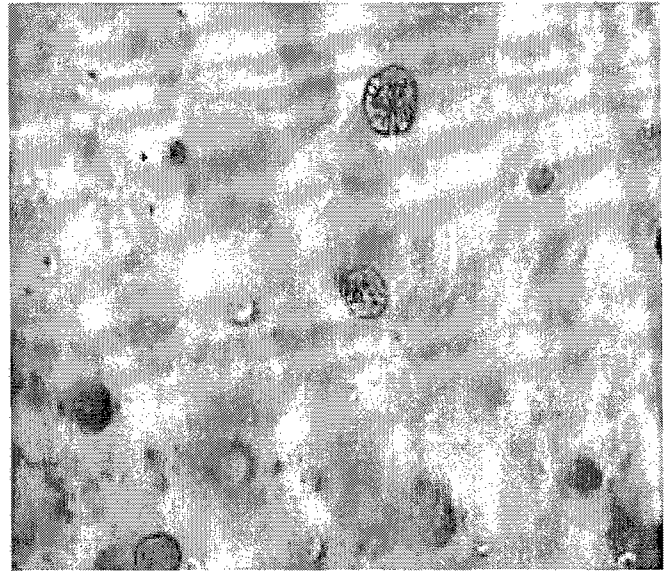
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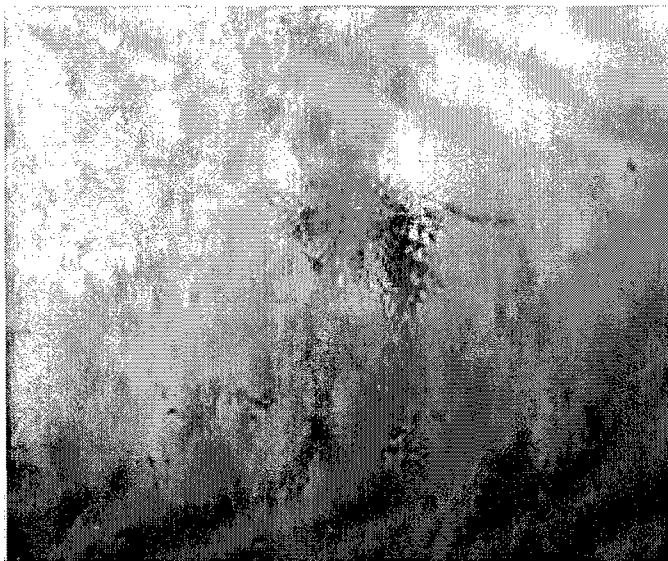
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MCF10A/pQ(4)



MCF10A/p65(1)



MCF10A/p65(4)

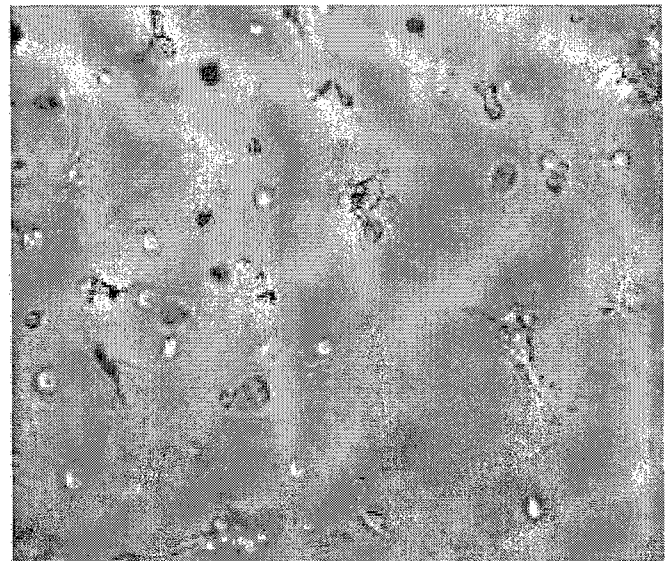
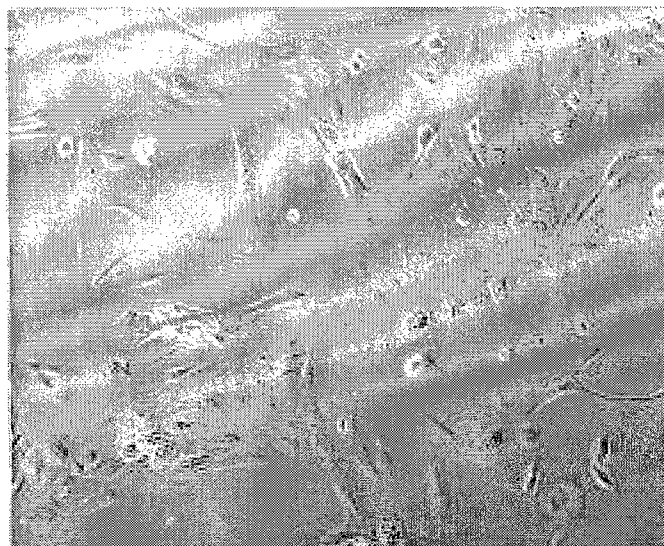


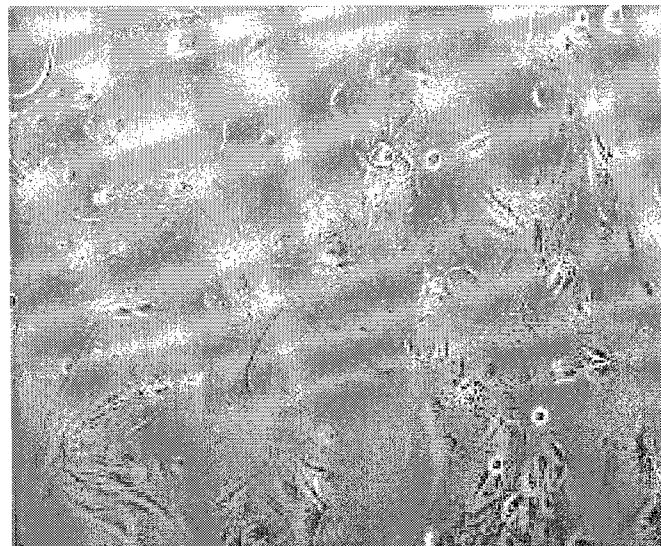
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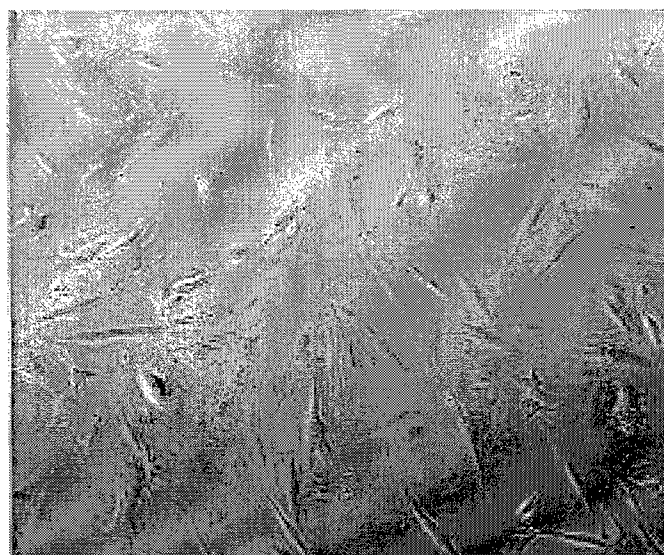
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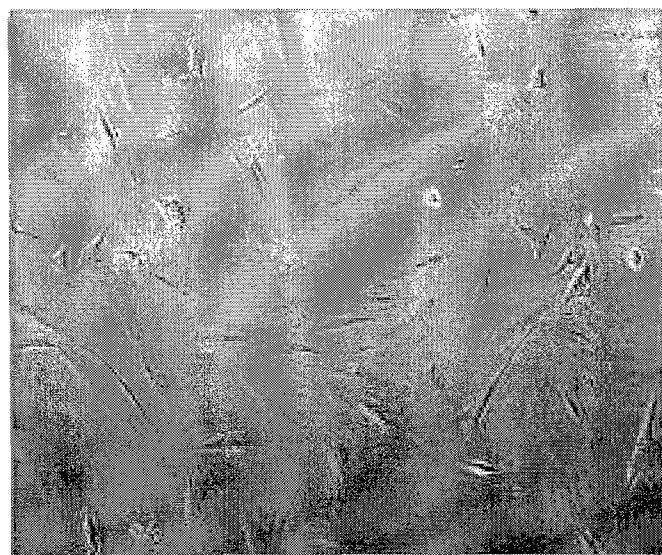
**MCF10A/pQ(4)**



**MCF10A/p65(1)**



**MCF10A/p65(4)**



**Figure 4A:** MCF10A/p65 cells show fibroblast morphology compared to MCF10A/pQ cells, which show epithelial morphology when grown on plastic.



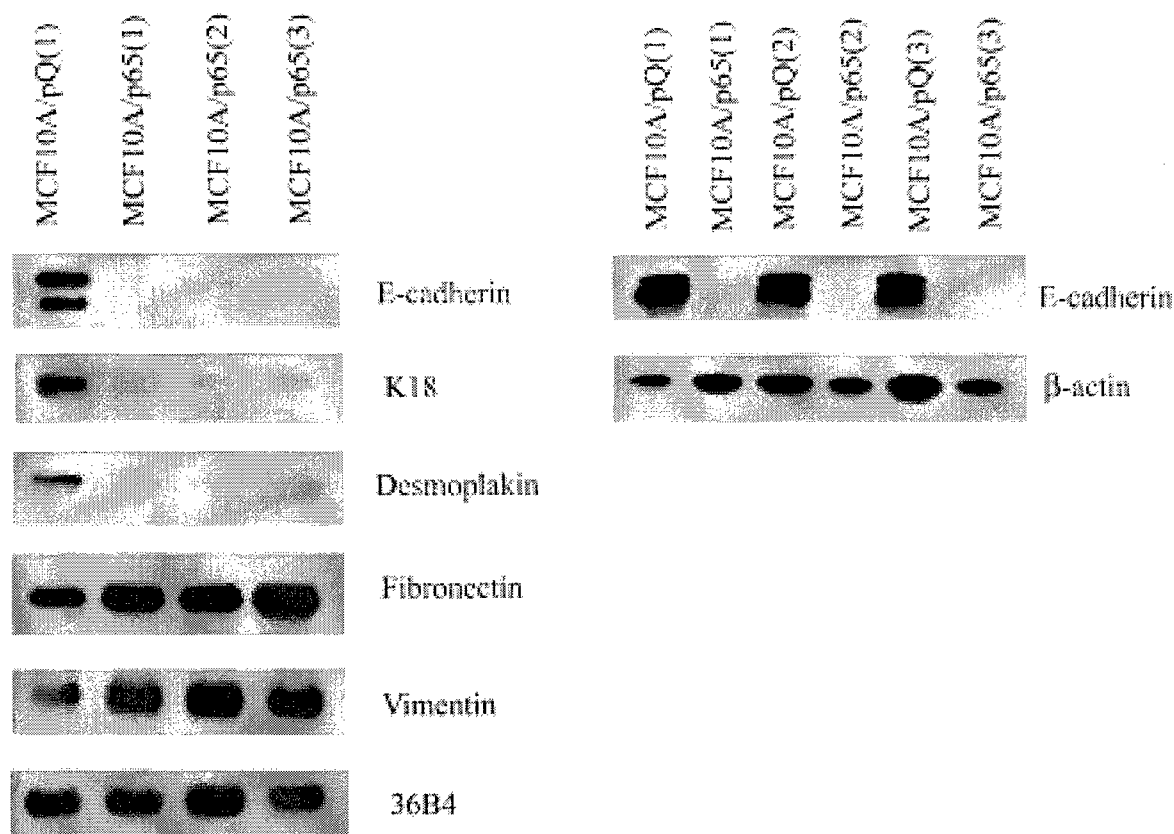


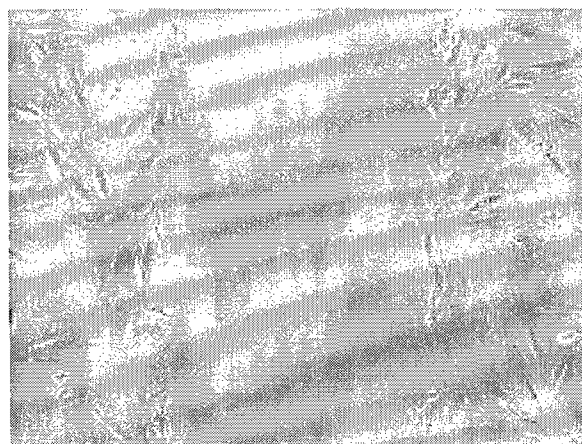
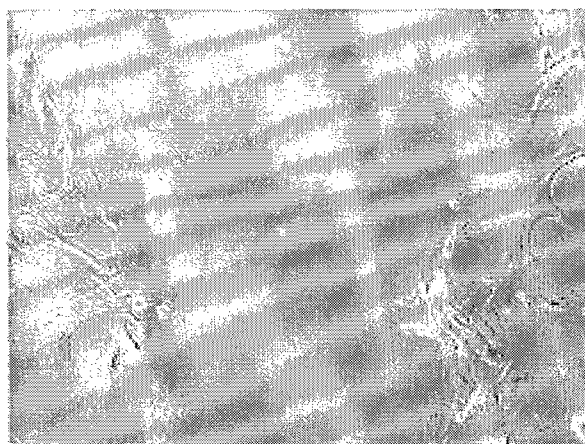
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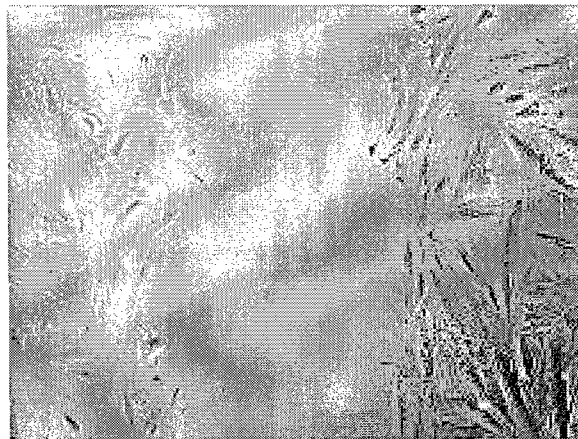
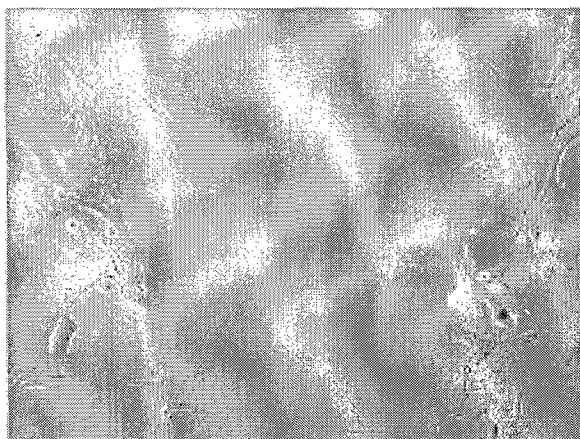
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**MCF10A/p65(1)**

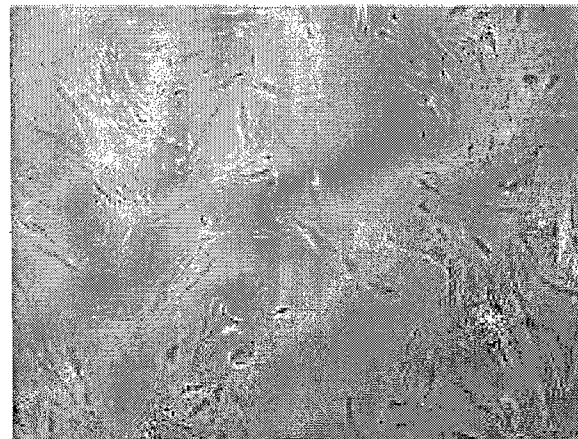
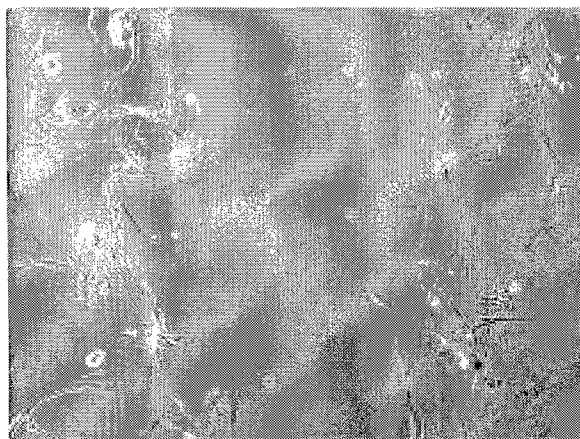
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**9 hr**



**24 hr**



**Figure 5: MCF10A/p65 cells show enhanced motility compared to MCF10/pQ cells. Cell motility was measured by wound assay.**



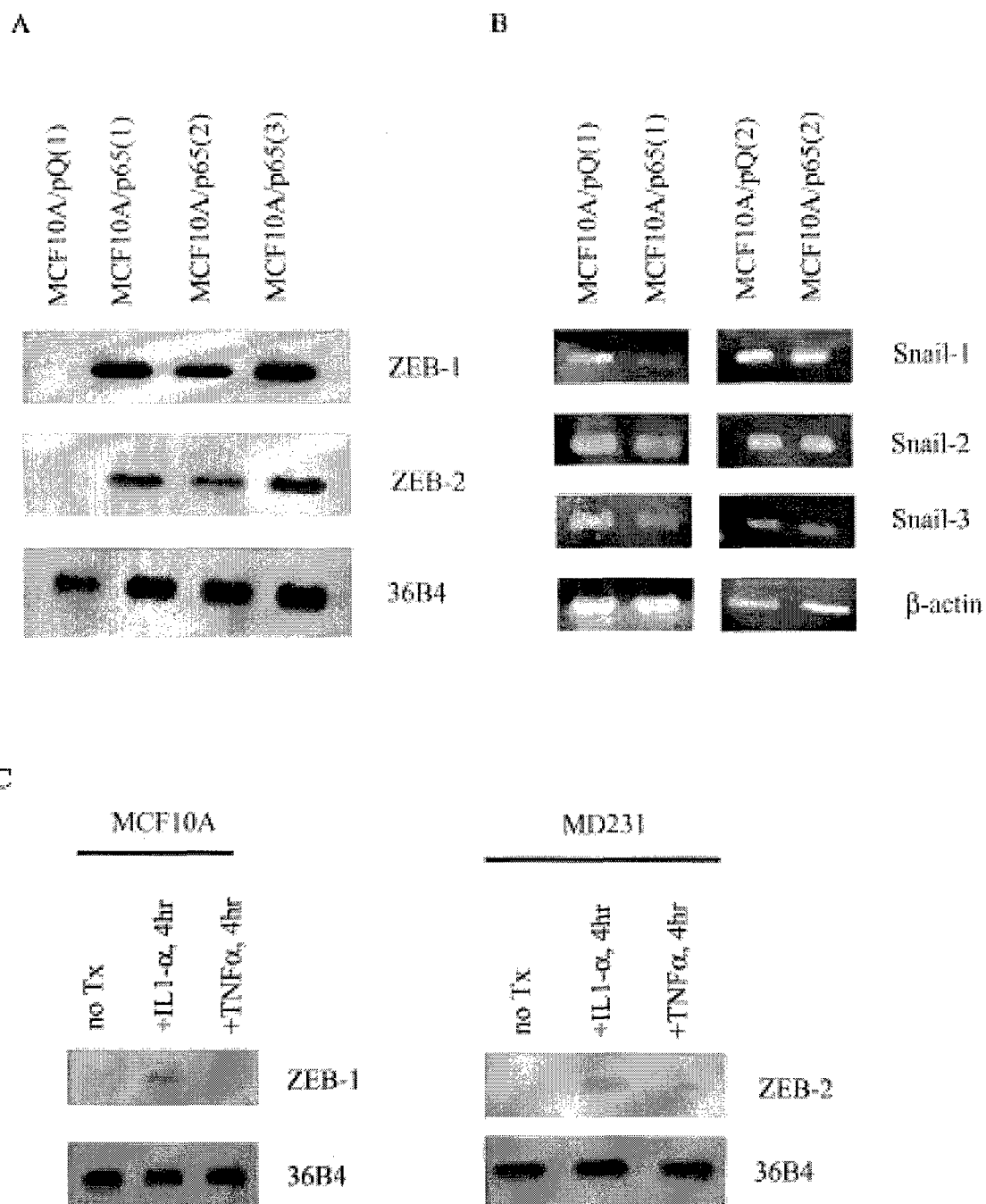
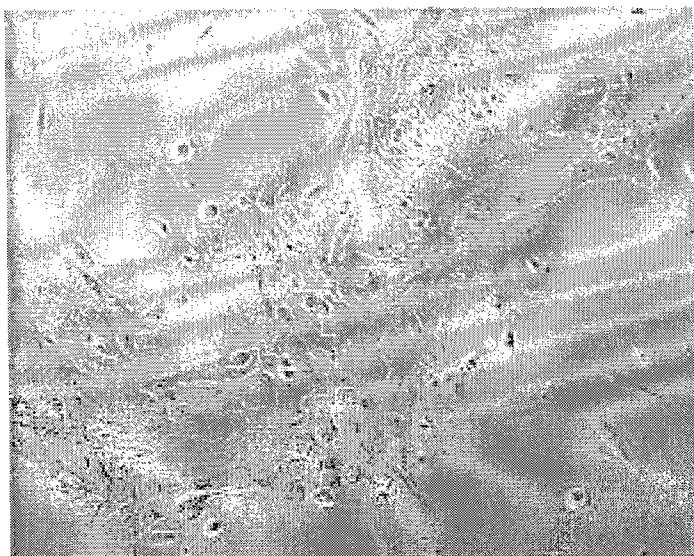


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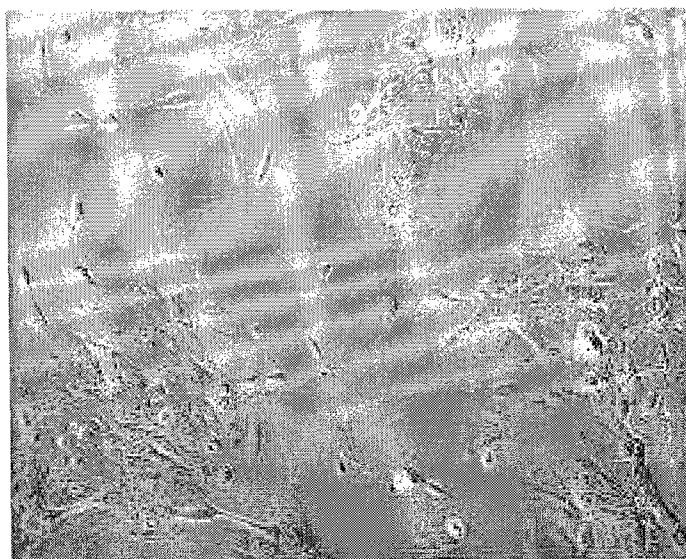


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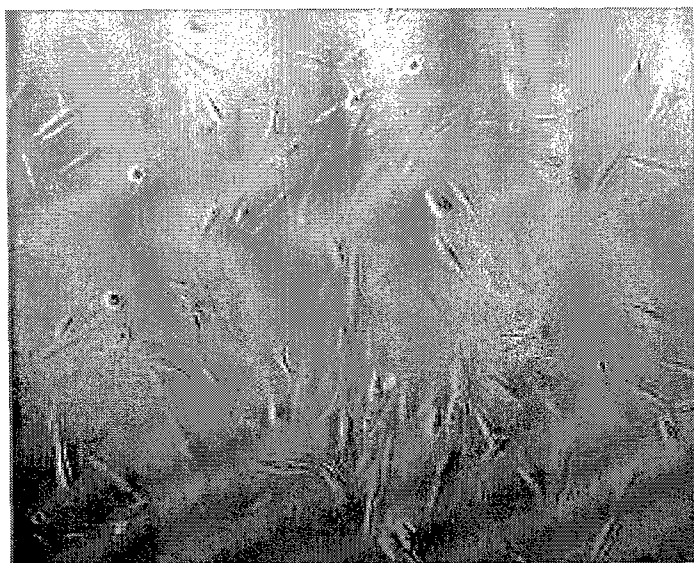
MCF10A/pQN(1)



MCF10A/pQN(2)



MCF10A/ZEB1(1)



MCF10A/ZEB1(2)

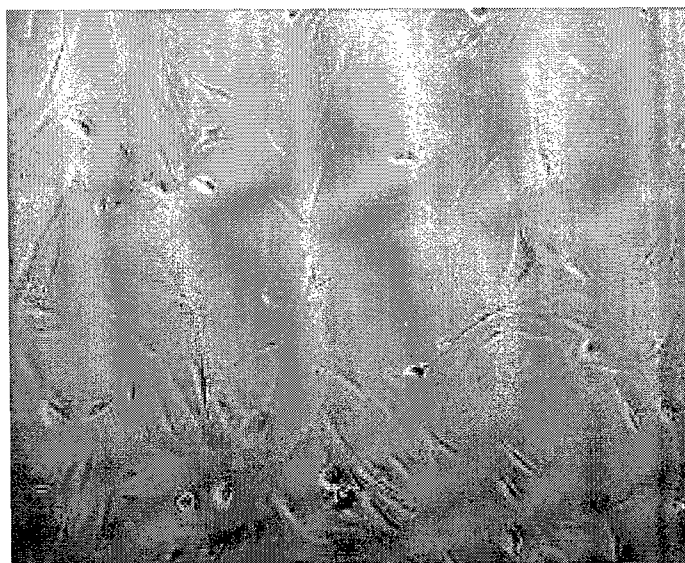


Figure 7A: MCF10A cells overexpressing ZEB-1 show fibroblastic morphology.



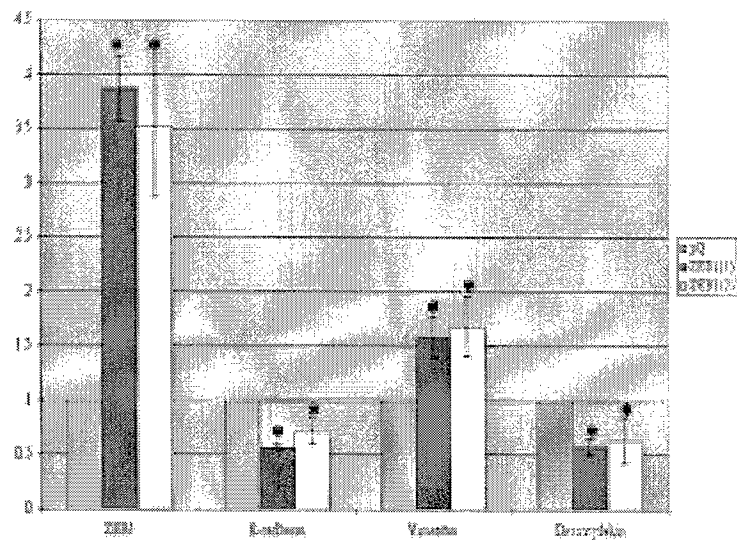
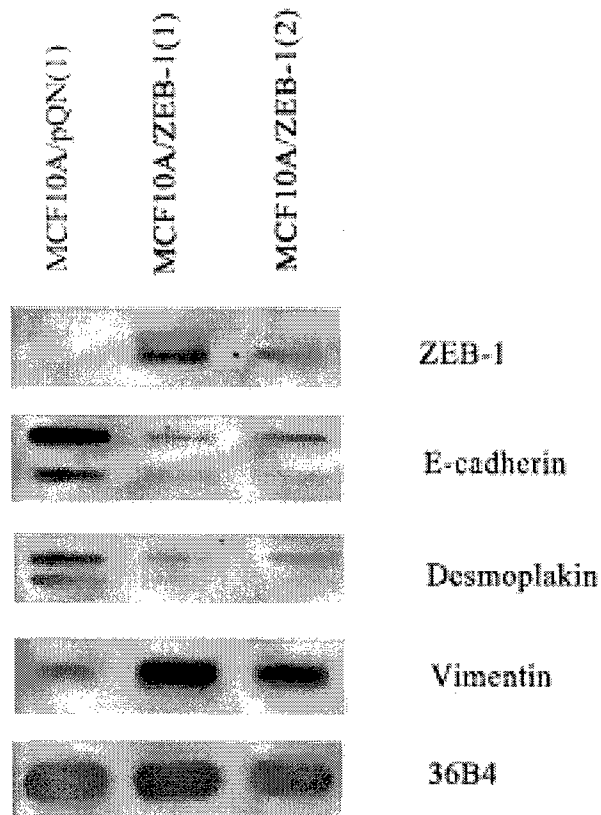


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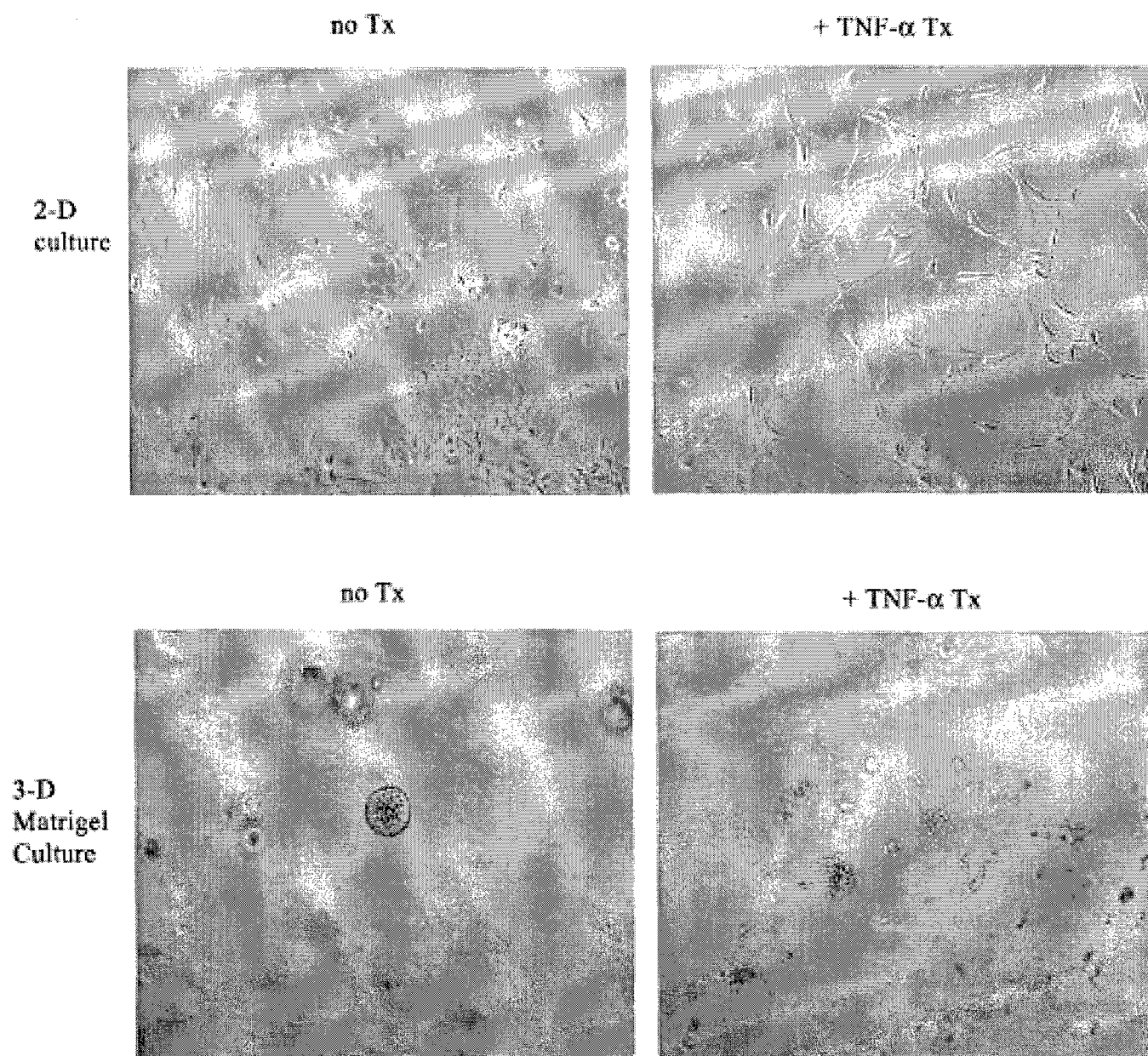


Figure 8: MCF10A cells exposed to TNF $\alpha$  for 22 days show fibroblastic morphology on plastic (top panel) and form a disorganized structure in matrigel (bottom panel).



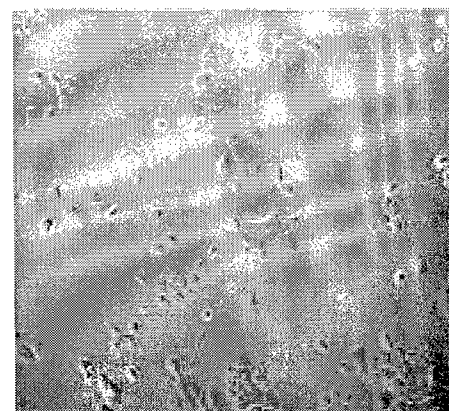
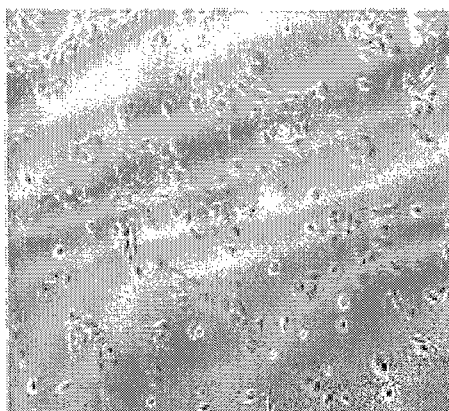
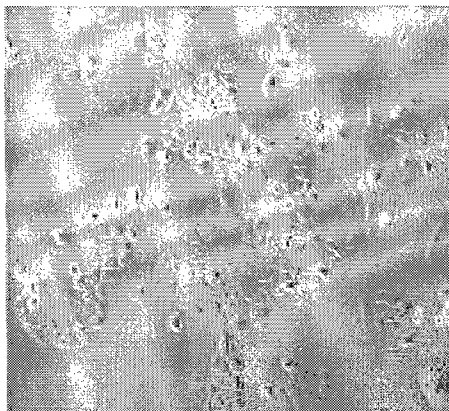
Days after  
TNF $\alpha$  removal

Day 11

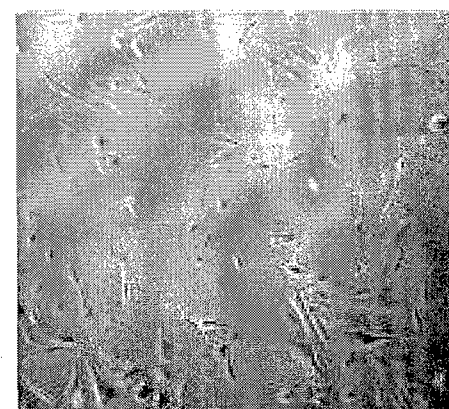
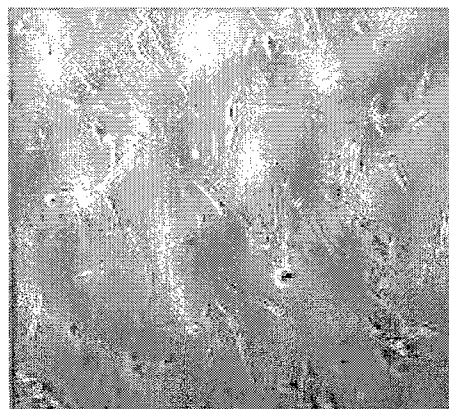
Day 15

Day 18

no Tx



TNF $\alpha$   
Tx



TNF $\alpha$   
removed

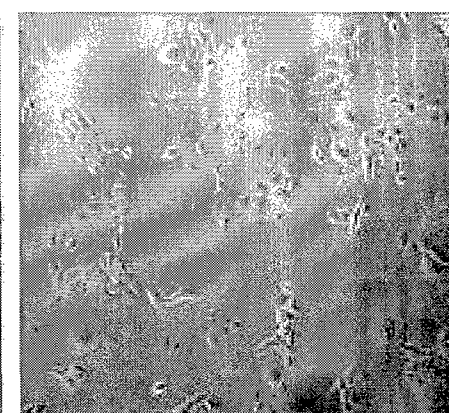
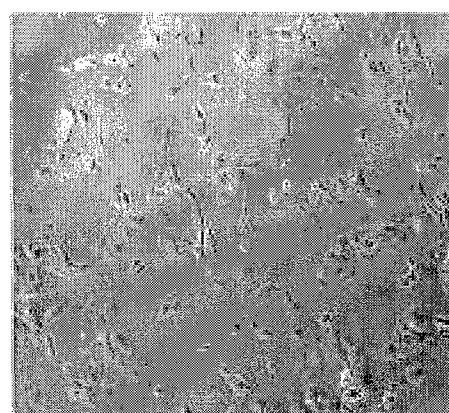
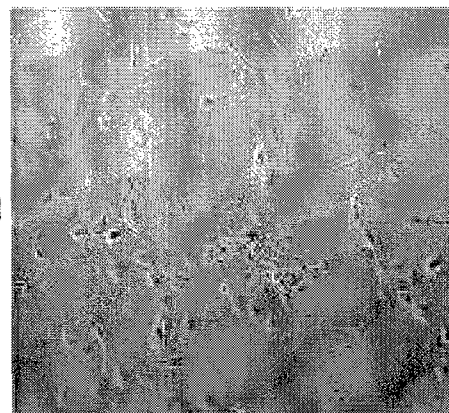


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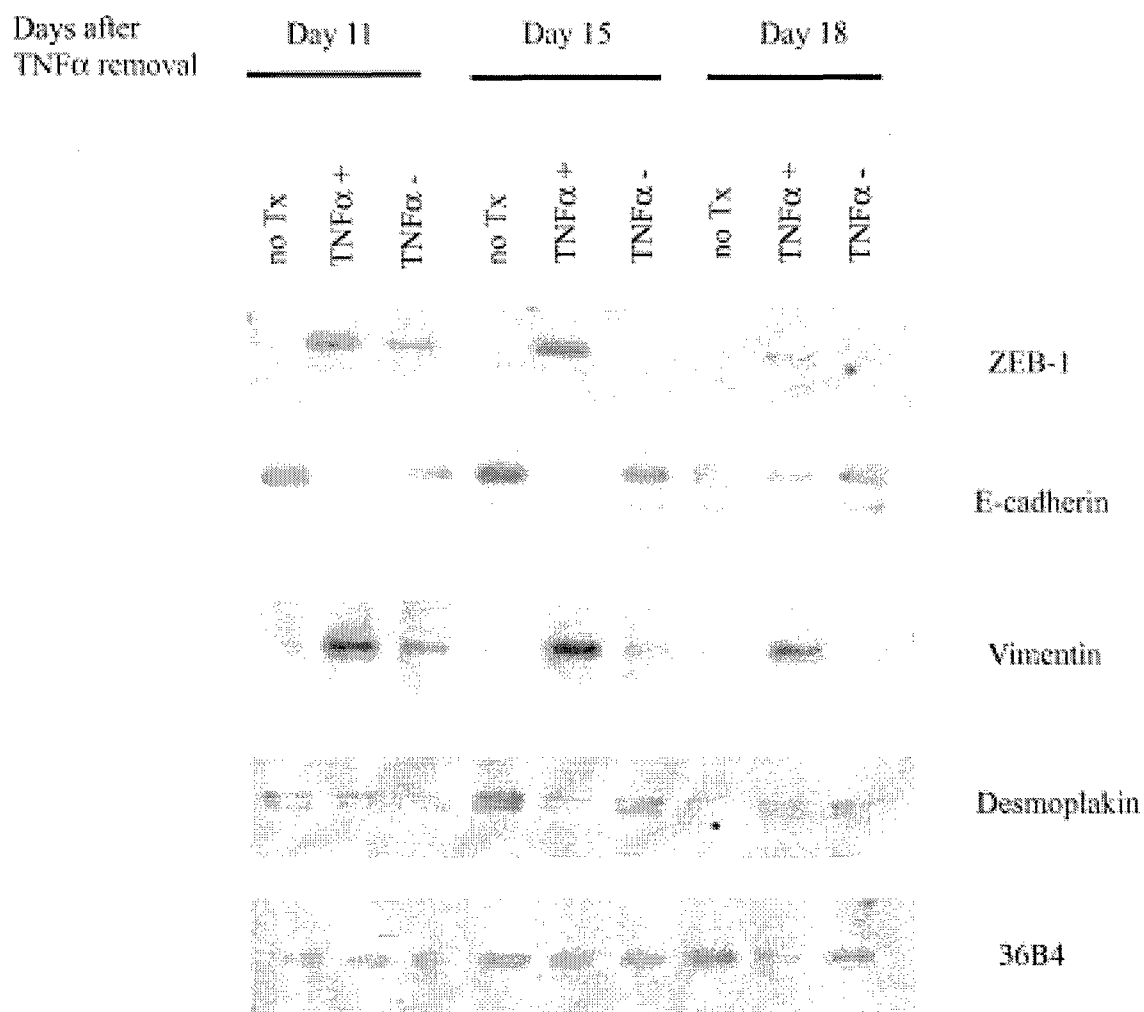


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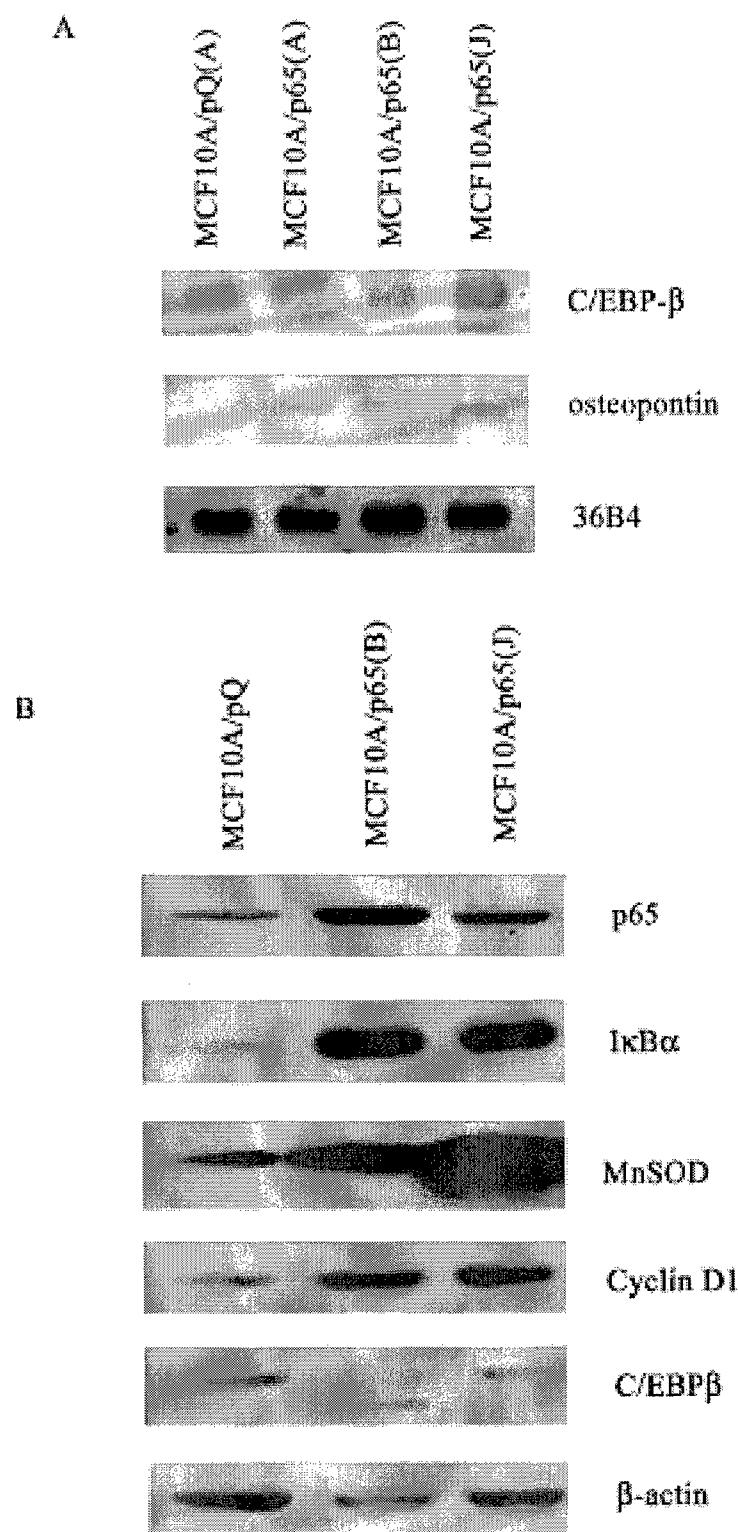


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C/EBP-C/EBP		C/EBP-GADD153		SP-1	
MCF10A/pQ(A)	MCF10A/p65(A)	MCF10A/pQ(A)	MCF10A/p65(A)	MCF10A/pQ(A)	MCF10A/p65(A)

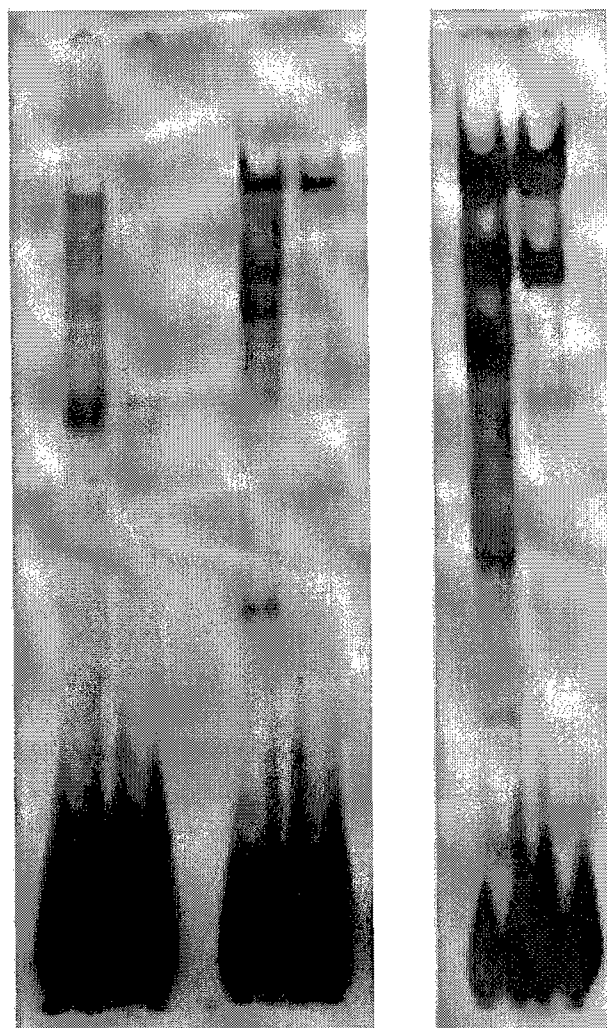


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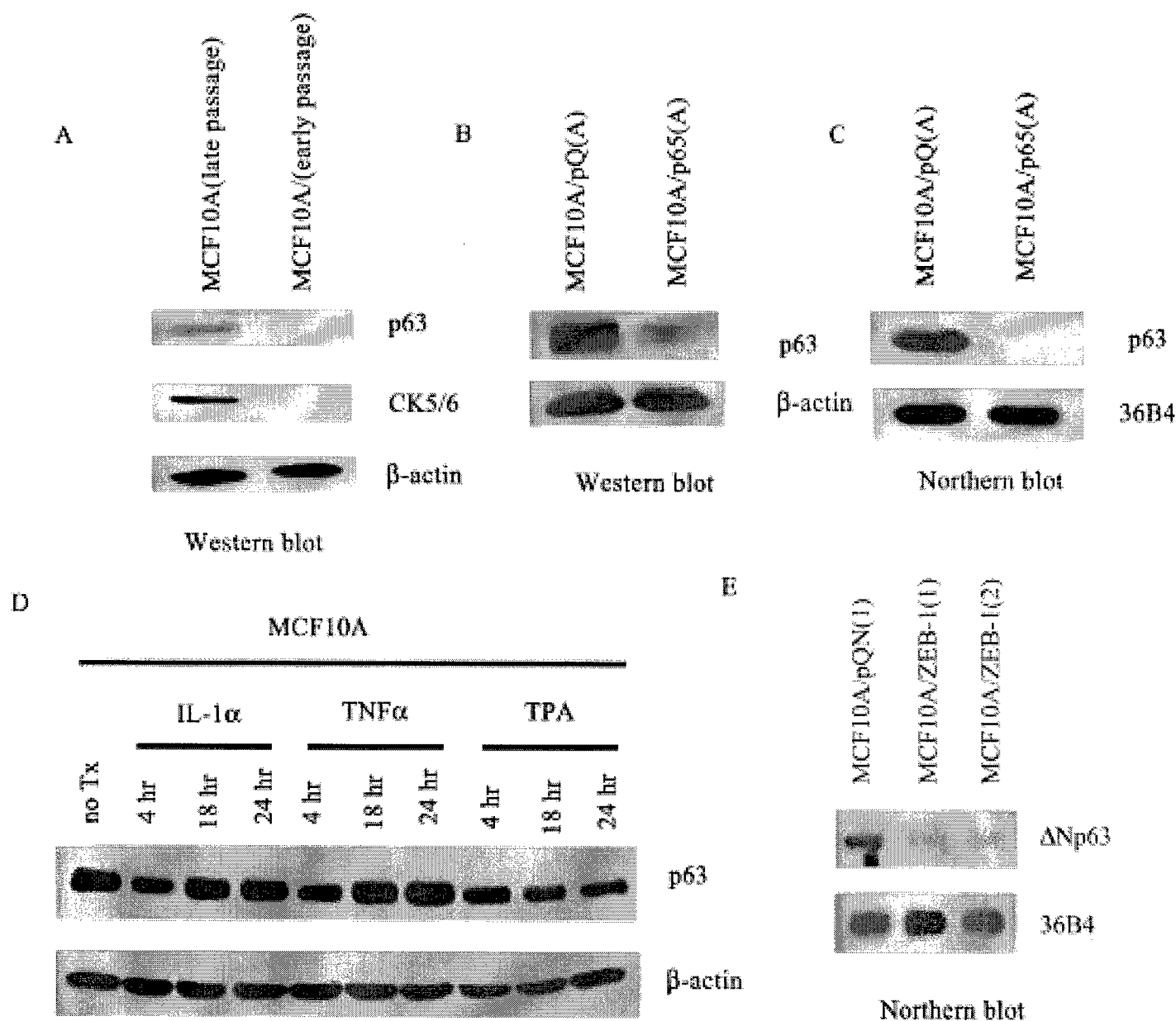


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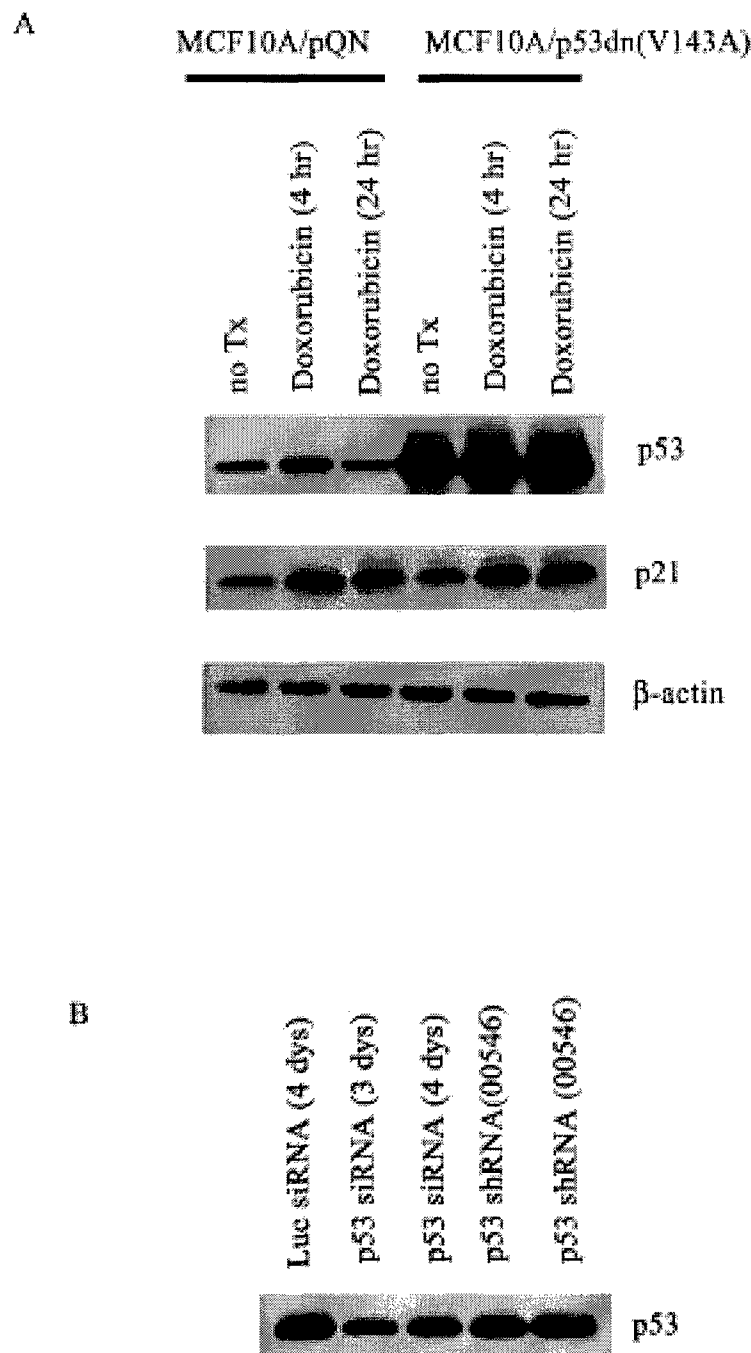


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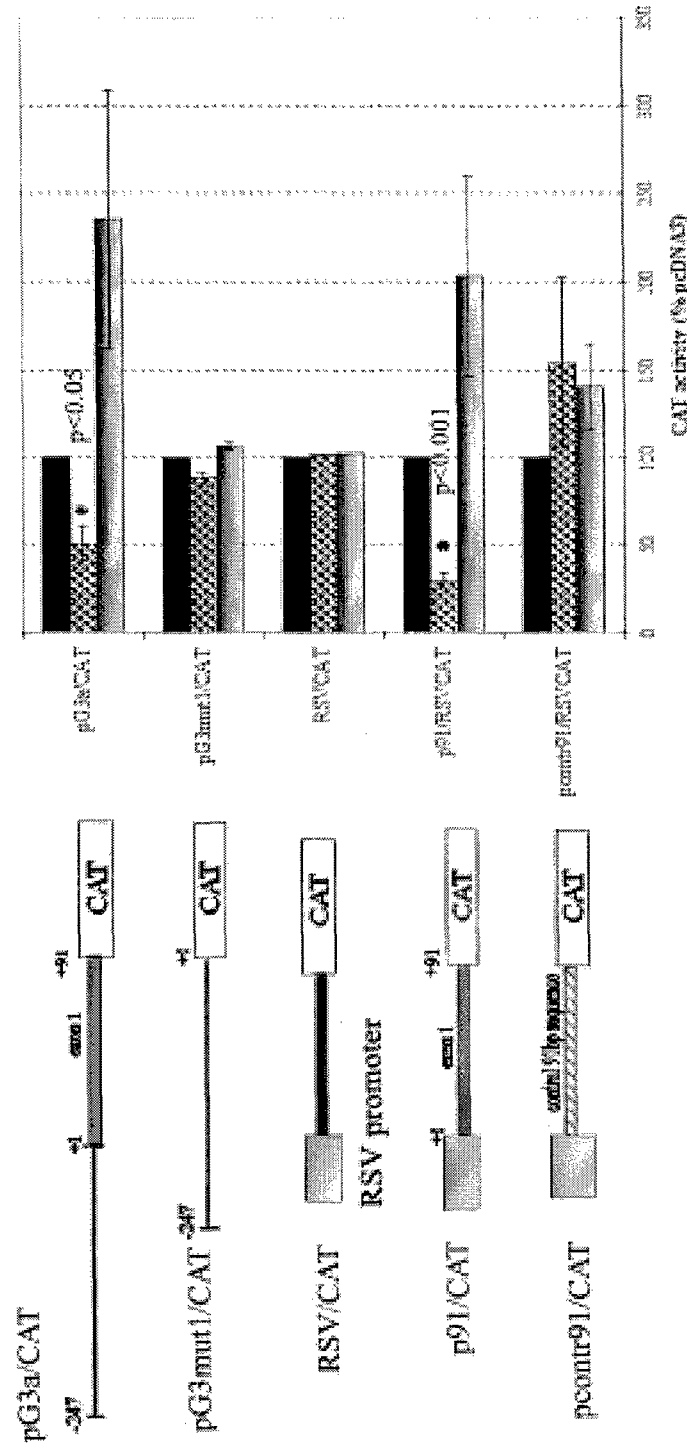


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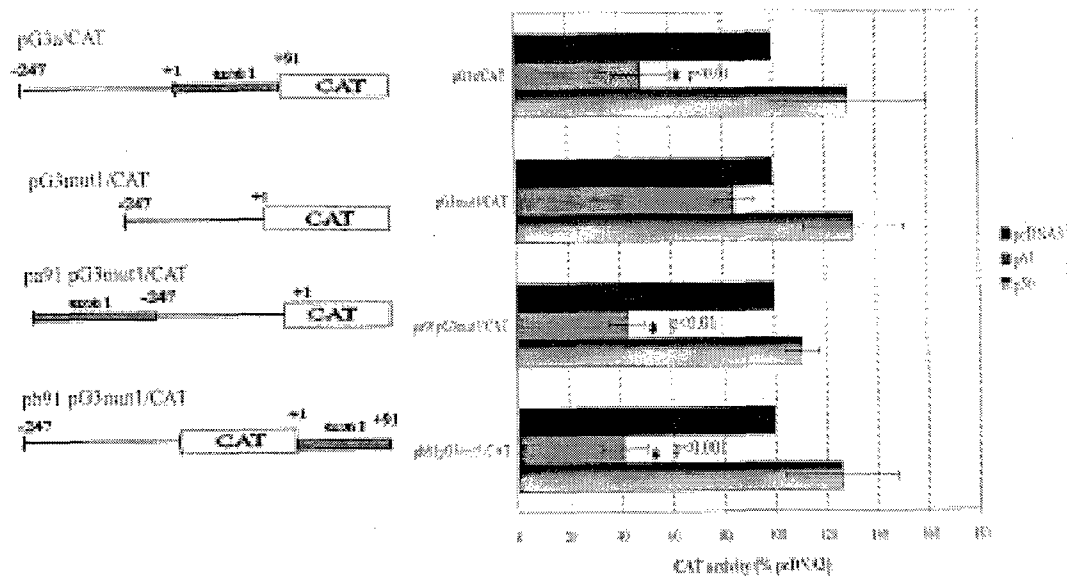


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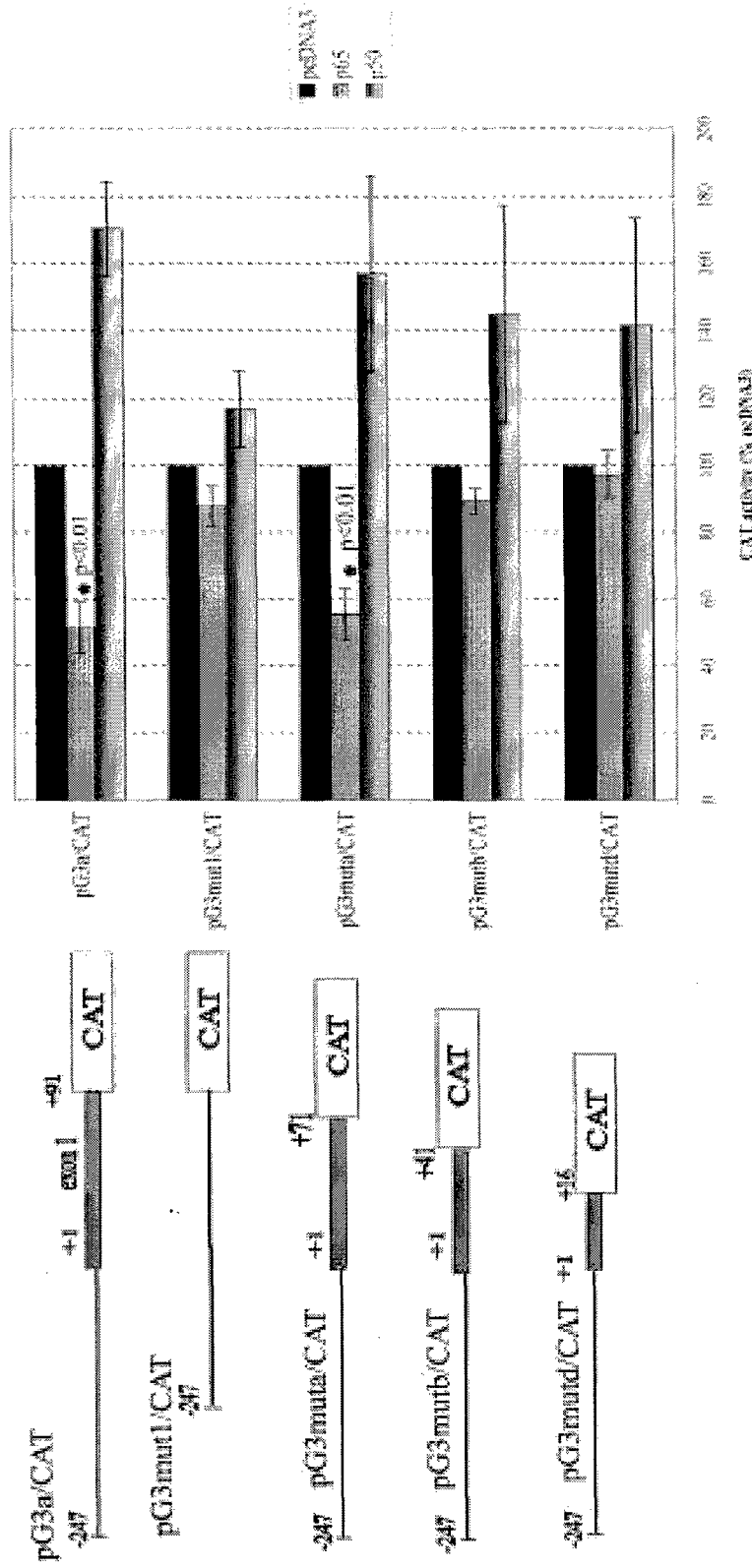


Figure 13C: pG3a/CAT reporter lacking sequences from +42 to +71 of GADD 153 (pG3mutb/CAT) was not repressed by p65 whereas pG3a/CAT reporter with +1 to +71 (pG3muta/CAT) was repressed by p65.



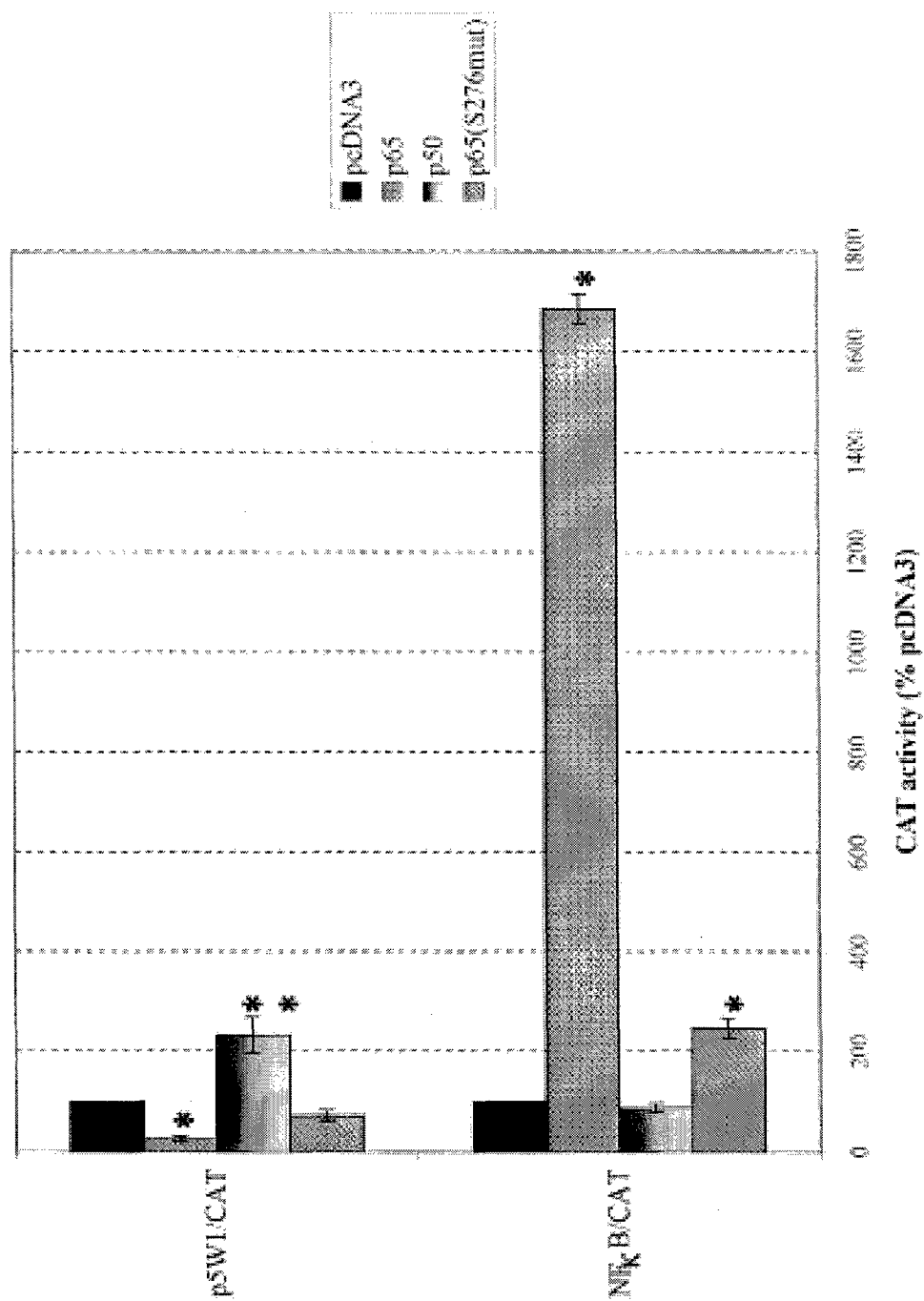


Figure 14: The p65 with mutation of S276 to alanine [p65(S276mut)] was less efficient than wild type p65 in repressing GADD153 promoter. P5W1/CAT corresponds to CAT reporter with -954 to +91 bases of GADD153 promoter. Note that p65S276mut is less efficient than wild type p65 in activating NFkB/CAT reporter.



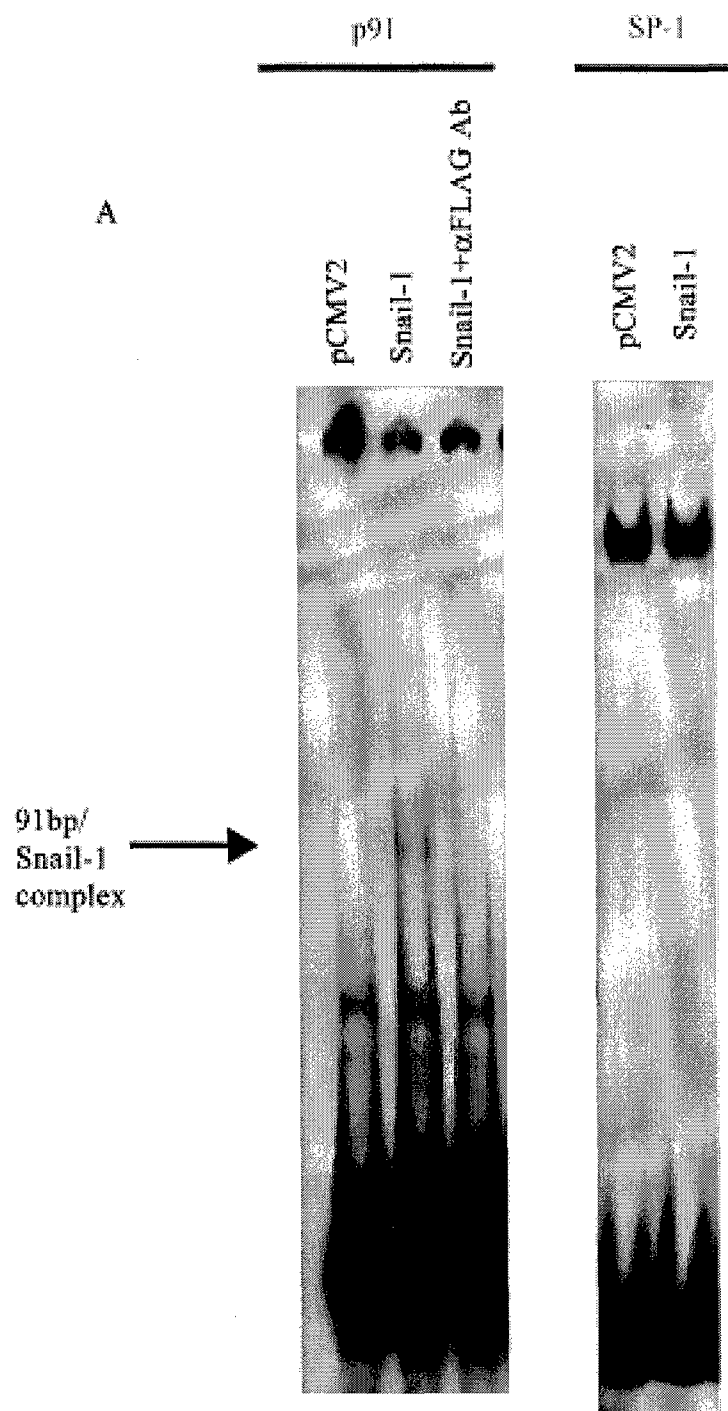


Figure 15A The exon 1 of GADD153 binds to Snail-1. Cos-1 cells were transfected with control plasmid pCMV2 or Flag-epitope tagged Snail-1. Radiolabeled 91 bp-untranslated region of exon 1 of GADD153 was incubated with nuclear extract from transfected cells and DNA: protein complex was resolved on an acrylamide gel. A specific DNA: protein complex was observed with nuclear extract from Snail-1 transfected cells. Flag antibody disrupted this complex.



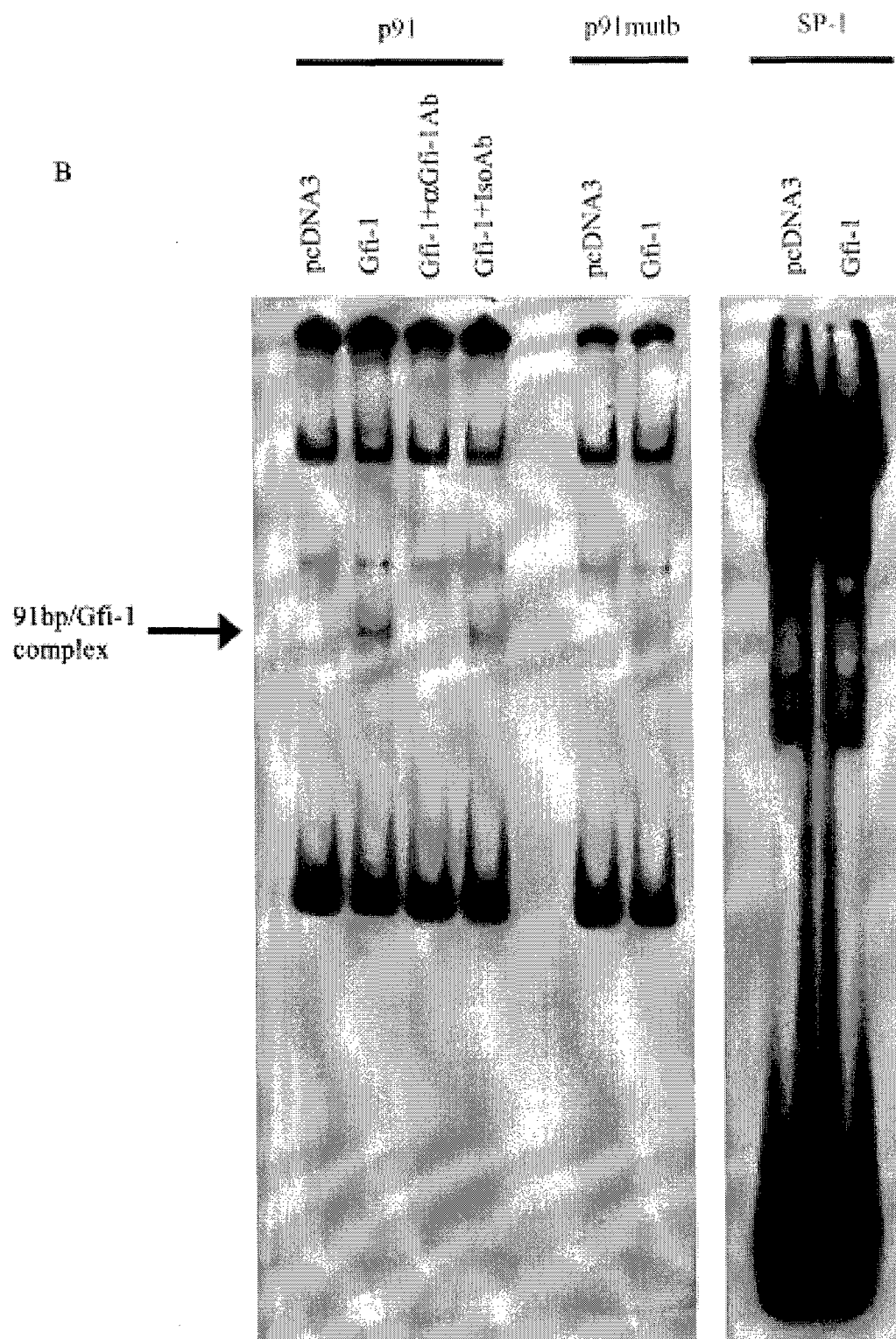


Figure 15B: Binding of Gfi-1 to exon 1 of GADD153. Experiments were done as in A. Gfi-1:DNA complex was detectable with nuclear extracts from cells transfected with gfi-1. This complex was disrupted by Gfi-1 specific antibody but not isotype control antibody. Also, binding of Gfi-1 to exon 1 with mutation of Gfi-1 binding site (p91mutb) was substantially lower compared to p91 probe.



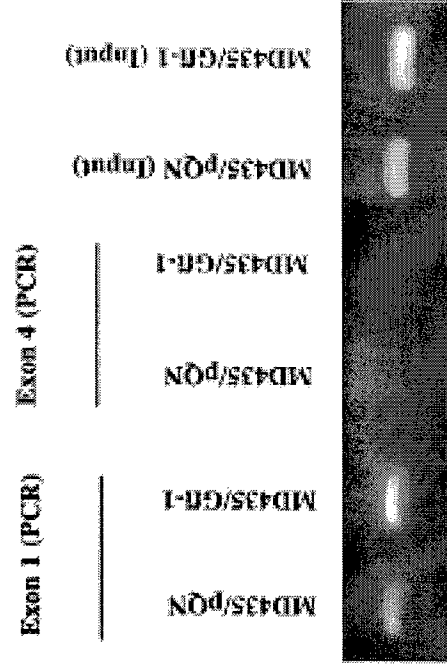


Figure 16: Binding of endogenous Gfi-1 to GADD153 promoter was determined by chromatin immunoprecipitation assay. MD435 cells were used because these cells express higher levels of Gfi-1. Gfi-1 immunoprecipitates from cross-linked cells contain sequences corresponding to exon 1 but exon 4 of GADD153. Gfi-1 binding was higher in cells that are overexpressing Gfi-1 (MD435/Gfi-1) compared to cells transfected with vector (MD435/pQN). PCR amplification of input DNA is also shown.



pG3a/CAT



pG3mutb/CAT

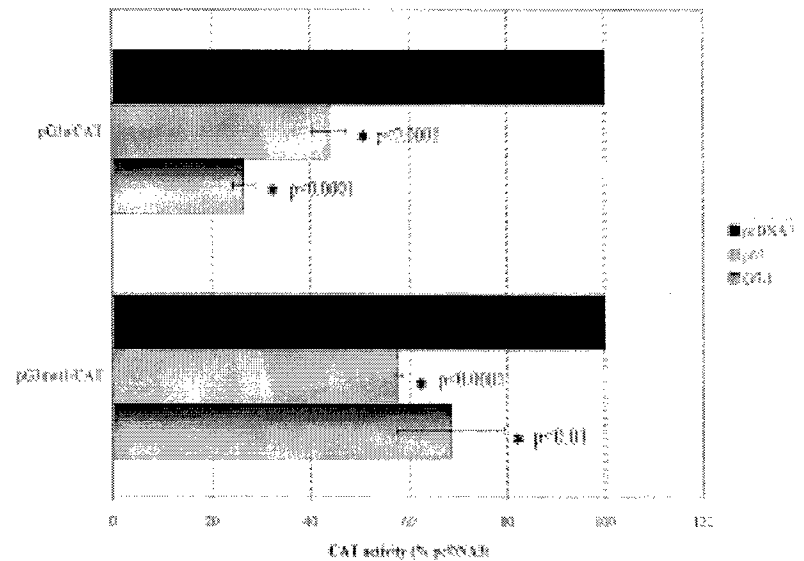
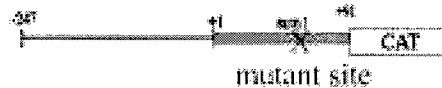


Figure 17: Gfi-1 represses GADD153 promoter. Cos-1 cells were transfected with pG3/CAT or pG3/CAT with mutation of Gfi-1 binding site (pG3mutb/CAT) and p65 or Gfi-1 expression vector. PCHI10 was used as an internal control to measure transfection efficiency. pG3a/CAT but not pG3mutb/CAT activity was lower in cells transfected with p65 or Gfi-1. Thus, Gfi-1 functions as repressor of GADD153.



# P59-14: NF-KAPPA-B PROMOTES EPITHELIAL-TO-MESENCHYMAL TRANSITION OF IMMORTALIZED MAMMARY EPITHELIAL CELLS THROUGH ZEB-1 AND ZEB-2

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Epithelial to mesenchymal transition (EMT) is a process by which epithelial cells acquire mesenchymal properties, enabling them to migrate to other environments. EMT therefore plays an important role in embryogenesis as well as in the growth, invasion, and metastasis of cancer cells. For example, EMT is observed in ~10% of breast cancer and EMT has recently been shown to generate nonalignant stroma which can support the growth of cancer cells. However, signaling pathways that induce EMT in breast cancer is not completely understood.

The extracellular signal activated transcription factor NF- $\kappa$ B has recently been identified as a major player in a variety of cancers. Constitutive activation of NF- $\kappa$ B has been observed during the early and hyperplastic stages of breast cancer. To understand the role of NF- $\kappa$ B in the early stages of breast cancer, the mammary epithelial cell line MCF10A overexpressing a constitutively active form of the p65 subunit of NF- $\kappa$ B was generated. The MCF10A/p65 cells acquired a fibroblastic morphology, which is characteristic of cells undergoing EMT. Parental MCF10A cells cultured in a three-dimensional matrix organized into polarized lobular acinar structures, whereas MCF10A/p65 cells formed disorganized structures, similar to fibroblasts. Transfected MCF10A cells described in other studies. MCF10A/p65 cells showed reduced expression of the epithelial markers E-cadherin, occludin, MUC1 and desmoplakin, whereas mesenchymal markers such as vimentin and fibronectin were increased. The expression of stromal markers like MMP3, which along with MMP13 promotes metastasis, was also increased in MCF10A/p65 cells. In addition, MCF10A/p65 cells showed an increase in cellular motility compared to parental MCF10A cells. Interestingly, the expression levels of the transcription repressors ZEB-1 and ZEB-2 but not Snail, all of which are involved in the repression of E-cadherin and induction of EMT, were elevated in MCF10A/p65 cells. The induction of ZEB-1 and ZEB-2 was also observed in a breast cancer cell line following NF- $\kappa$ B activation. These results suggest that NF- $\kappa$ B promotes breast cancer initiation through EMT and ZEB-1 and ZEB-2 may mediate EMT in breast cancer cells with constitutively active NF- $\kappa$ B.

Original work supported by the U.S. Army Medical Research and Materiel Command under DAMRD17-01-1-0374

# P59-15: THE ROLE OF CXCL12 AND CXCL14 CHEMOKINES IN EPITHELIAL STROMAL CELL INTERACTIONS DURING BREAST TUMOR PROGRESSION

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Despite compelling cell biological studies and histopathological observations implicating stromal cells in tumorigenesis, our knowledge of the genes that mediate stromal changes and interactions among various cell types in breast cancer, and their role in tumor initiation and progression is limited. Similarly the occurrence and role of genetic changes in stromal cells are undefined. In order to gain insight into these questions, we analyzed the comprehensive gene expression profiles of each cell type comprising normal breast tissue and in situ and invasive breast carcinomas using Serial Analysis of Gene Expression. Based on these data we determined that extensive gene expression changes occur in all cell types during cancer progression and a significant fraction of altered genes encode secreted proteins and receptors. Despite the dramatic gene expression changes in all cell types, genetic alterations were only detected in cancer epithelial cells. The CXCL14 and CXCL12 chemokines overexpressed in tumor myoepithelial cells and myofibroblasts, respectively, bind to receptors on epithelial cells and enhance their proliferation, migration, and invasion. Thus chemokines may play a role in breast tumorigenesis by acting as paracrine factors. To test this hypothesis we generated derivatives of breast cancer and myoepithelial cell lines that overexpress these chemokines or downregulated their expression using shRNA. These cells were then analyzed in various *in vitro* and *in vivo* assays to determine the effect of chemokines on cellular growth, invasion, and tumorigenesis. Our data support the hypothesis that the CXCL12 and CXCL14 chemokines act as paracrine factors and influence breast tumorigenesis, suggesting that the therapeutic targeting of these pathways may be a new approach for breast cancer treatment.

The U.S. Army Medical Research and Materiel Command under W81WH06-2-0043 supported this work

# P59-16: ALTERED BREAST CELL PHENOTYPE, SIGNALING AND GENE EXPRESSION BY PHYSICAL PROPERTIES OF 1 EXTRACELLULAR MATRIX

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Dense breast tissue is linked to a greater than four-fold increased risk of breast cancer, a relative risk greater than almost all other risk factors for breast cancer. As up to a third of breast cancer cases may be related to dense breast tissue, and hormone replacement therapy is known to increase breast density, importantly, increased breast density are associated with significantly increased collagen deposition. Although the mechanisms driving the effect of the extracellular matrix (ECM) on breast cancer development are largely not known, one contributing factor may be physical signals imposed on mammary epithelial cells from surrounding type-1 collagen-rich stroma either directly or across basement membrane proteins.

Mammary cells (MMMG) display altered morphology and adhesion signals particularly focal adhesion kinase (FAK) phosphorylation, when cultured in cells matrices with altered physical properties or with tensile loading of the ECM. MMMG cells cultured in type-1 collagen gels form a similar model system of the neonatal mammary gland as they organize the type-1 collagen matrix and deposit the basement membrane proteins laminin 1, 2, and type-IV collagen surrounding ductal and alveolar structures in *in vivo* conditions. In contractile collagen gels, MMMG cells form ductal structures similar to those seen in human breast cells, however in stiffer matrices or non-contractile gels, tubulogenesis is not fully disrupted but instead ductal structures are present with altered morphology. Ducts in stiff matrices are shorter and play membrane protrusions that possess 3D adhesions containing localized vinculin, paxillin, as well as stress-fiber-like actin structures. Additionally, these 3D adhesions in stiff matrices contain localized FAK (Y397), Src (Y416), and paxillin (Y31). On genetic level, microarray analysis reveals a large number of differentially expressed genes (~1400) in denser matrices, with families of genes associated with proliferation, survival, migration, adhesion, ECM composition and degradation, signaling, and cytoskeleton revealing an expressive pattern indicative of a more proliferative invasive phenotype in denser matrices.

The estrogen receptor (ER) positive T47D breast carcinoma cell line forms well-defined ductal structures in contractile collagen gels of moderate density, while it in non-contractile "fixed" gels or high density (higher elastic modulus) collagen gels have disrupted tubulogenesis. In T47D cells, initial microarray experiments indicate increased expression of estrogen responsive genes in cells cultured within 3D matrix. Additional analysis revealed that ER- $\alpha$  expression is increased for matrix conditions in which tubulogenesis is optimal, while denser matrices reduce ER- $\alpha$  mRNA levels. This trend is additionally evident for the estrogen responsive progesterone receptor and cathepsin-D genes, with silencing of anti-estrogen receptor progesterone receptor's dependent on ER- $\alpha$ . Combined, these results begin to elucidate some of the mechanisms that may be associated with increased ductal risk and poor tumor formation and progression in cancers associated with dense breast tissue.

The U.S. Army Medical Research and Materiel Command under W81WH06-2-0043 supported this work

# P59-17: STROMAL-EPITHELIAL INTERACTIONS, MAMMARY TISSUE ARCHITECTURE AND TUMOR ANGIOGENESIS

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Introduction: Tumor metastasis depend upon the loss of tissue architecture and acquisition of tumor invasion and angiogenesis. Malignant transformation of the breast is associated with induction of a wounding response characterized by fibroblast invasion, collagen deposition and increased expression of reactive stromal integrins such as  $\alpha 5$  and  $\alpha 6$  promote malignant transformation and breast tumor metastasis by compromising mammary tissue integrity and promoting angiogenesis.

Experimental Approach: We used the HMT1232 breast cancer progression cell line which consists of non-malignant 81 cells, pre-malignant 53 cells and malignant T-cells in conjunction with reconstituted basement membrane (RBM) to culture a xenograft studies. Integrin expression profiles and levels of pro-angiogenic factors were assessed using flow cytometry, immunoblot and ELISA assays. Phenotypic angiogenic behavior of the 81, 53 and T42 mammary epithelial cells (MECs) was monitored using RBM cultures, soft agar assay, Boyden chambers and three dimensional (3D) RBM-collagen co-culture with MECs and human vascular endothelial cells (HMECs) and by xenograft studies. To determine whether expression of active stromal integrins such as  $\alpha 5$  and  $\alpha 6$  promote malignant behavior of MECs, using



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#### An NF- $\kappa$ B-repressible element in the 5'-untranslated region of the pro-apoptotic GADD153/CHOP gene

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Cells exposed to stresses such as nutrient deprivation, hypoxia, ultraviolet light and agents that damage DNA or disrupt endoplasmic reticulum function show induced expression of GADD153/CHOP. GADD153 is a member of the C/EBP family of transcription factors and promotes apoptosis following cellular stress. Interestingly, cellular stress also induces NF- $\kappa$ B, which promotes cell survival by inducing transcription of a number of anti-apoptotic proteins, such as Bcl-2, Bcl-XL, x-IAP and c-IAP-2. GADD153 mRNA expression was previously shown in this laboratory to be inhibited by the p65 transactivating subunit of NF- $\kappa$ B. Therefore, cells expressing constitutively active NF- $\kappa$ B, as is the case in 30% of breast cancers, may be predisposed to transformation or malignancy, due to p65-mediated inhibition of GADD153 expression following cellular stress. By transient transfection assays, we identified an NF- $\kappa$ B-repressible element (NRE) within a 91-base pair portion of the 5'-untranslated region of the GADD153 gene. In addition, transient transfection of p65 with various co-activators or co-repressors failed to reverse the inhibitory effect. Insertion of GADD153 NRE, but not an unrelated 91-base pair sequence, to the 5'-untranslated sequence of the Rous Sarcoma Virus enhancer/promoter-CAT reporter (RSV/CAT) led to p65-dependent repression of RSV/CAT expression. Interestingly, the same region of GADD153 has been previously shown to be required for UVC-mediated repression of stress-inducible GADD153 expression. We did not observe direct binding of the p65 subunit or any p65-inducible proteins to the 91-base pair DNA sequence by electrophoretic mobility shift assay. Based on a previous observation of MyoD mRNA destabilization by p65, we speculate that a p65-inducible protein destabilizes GADD153 mRNA by binding directly to the 5'-untranslated region of GADD153 mRNA. Understanding the mechanisms by which NF- $\kappa$ B mediates inhibition of GADD153 expression will facilitate the design of strategies to promote apoptosis of damaged cells thereby preventing initiation of transformation.

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## NF-κB and Breast Cancer

**B**reast cancer is the leading cause of death among women in North America, with a 12% lifetime risk.<sup>1</sup> Although there has been considerable progress in early detection and treatment, about 50% of patients eventually die of cancer.<sup>2</sup> Death in most cases is due to metastasis and resistance to chemotherapy. Understanding the mechanism of metastasis and chemoresistance is critical for developing molecular target-based therapies for breast cancer.

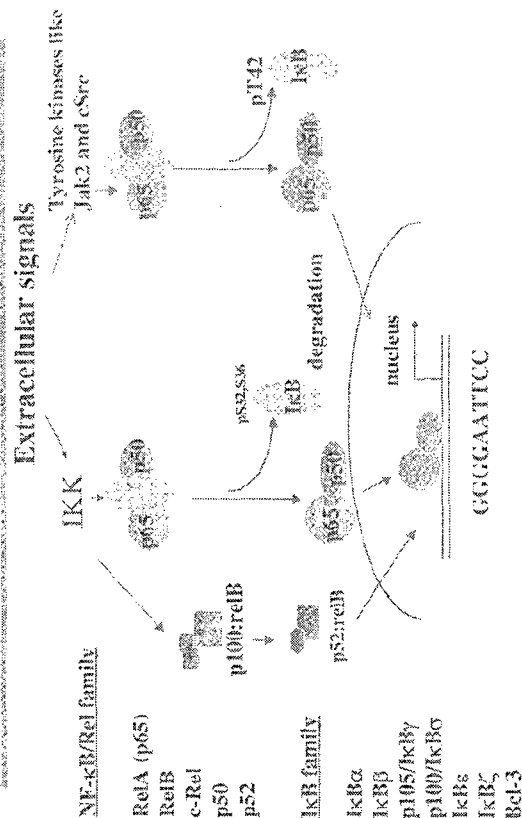
Recent developments in microarray technology have enabled molecular profiling of cancers. It is proposed that molecular profiling will offer more accurate predictions of future metastasis and treatment options.<sup>3-4</sup> Molecular profiling also permits selection of targets for therapy. Iressa, Gleeevec, and Herceptin are some of the examples that are targeted against epidermal growth factor receptor (EGFR), Bcr-Abl, and the her2/Neu oncogene, respectively.<sup>5-7</sup> Unfortunately, most cancers undergo multiple changes, which makes it difficult to design molecular target-based therapy. An alternative approach to these cancers is to identify and target transcription factors that control the expression of genes involved in cancer initiation, progression, metastasis, and chemoresistance. In this article, we discuss the specific roles of nuclear factor-κB (NF-κB), a transcription factor, in the regulation of genes involved in tumorigenesis and chemoresistance. We also highlight recent progress in the development of inhibitors of NF-κB as chemosensitizers for breast cancer.

### NF-κB

NF-κB was first identified in 1986 as the transcription factor involved in B lymphocyte-specific expression of κ-immunoglobulin light chain.<sup>8</sup> DNA binding activity was not detected in the nuclei of pre-B cells but could be activated by a posttranslational mechanism in these cells. Subsequent studies revealed ubiquitous activity of NF-κB and >180 target genes, including a variety of cytokines, cytokine receptors, cell adhesion molecules, and cell cycle regulatory and antiapoptotic genes.<sup>9,10</sup> Unlike general transcription factors, NF-κB activity in normal cells is

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## TRANSCRIPTION FACTOR NF-κB



**Fig 1.** Three distinct mechanisms of NF-κB activation: Extracellular signals, which include growth factors, cytokines, chemotherapeutic drugs, and oxidative stress activate IKK or a tyrosine kinase cascade leading to unmasking of nuclear localization signal and nuclear translocation of NF-κB.

tightly controlled, regulated primarily at the level of cytoplasmic and nuclear localization. A schematic view of this regulation is depicted in Fig 1.

The NF-κB family consists of five members, each with a unique 300-amino acid-long dimerization domain called the *rel* homology domain. This domain is required for dimerization, nuclear translocation, and DNA binding.<sup>9</sup> Members of the NF-κB family include NF-κB1 (p105/p50), NF-κB2 (p100/p52), *RelA* (p65), *RelB*, and *c-Rel*. Although the p65:p50 heterodimer is the most predominant, several distinct cell type-specific homodimeric and heterodimeric complexes of NF-κB have been identified.<sup>11</sup> In resting cells, with the exception of mature B cells, NF-κB is sequestered in the cytoplasm through association with a protein inhibitor-of-κB (IκB). There are six IκB proteins, which includes IκBα, IκBβ, IκBγ, IκBε, IκBζ, and *Bcl-3*.<sup>12-14</sup> The highly conserved sequence called ankyrin repeats of IκB mask the nuclear localization signal of NF-κB, thus retaining them in the cytoplasm. The p105 and p100



proteins, which are precursors for p50 and p52, respectively, have similar ankyrin repeats and function similar to I $\kappa$ B before processing.<sup>12</sup>

The mechanism of NF- $\kappa$ B activation has been studied extensively and described in several recent reviews.<sup>15-20</sup> Briefly, NF- $\kappa$ B is activated by a number of stimuli, which includes cytokines (tumor necrosis factor [TNF] family including receptor activator of NF- $\kappa$ B ligand [RANKL], interleukin [IL]-1, IL-17, IL-18), growth factors (EGF, PDGF, EPO, insulin, heregulin), physiologic mediators (angiotensin II, platelet activating factor), bacterial infection (*Staphylococcus aureus*), viral infection (HIV-1), bacterial and viral products (lipopolysaccharide, hemagglutinin), reactive oxygen species (hydrogen peroxide), stress (UV irradiation, pH, hypoxia, heavy metals), apoptosis mediators (anti-Fas), and chemotherapeutic drugs (taxol, doxorubicin, camptothecin).<sup>16,17</sup> These inducers activate at least three distinct pathways leading to activation of NF- $\kappa$ B (Fig 1). The majority of the inducers activate the I $\kappa$ B kinase complex (IKK), which phosphorylates I $\kappa$ B $\alpha$  at serine residues 32 and 36. Phosphorylated I $\kappa$ B $\alpha$  is degraded by the 26S proteasome leading to the unmasking of a nuclear localization signal of the p65:p50 heterodimer.<sup>17,20</sup> The second pathway involves phosphorylation of I $\kappa$ B $\alpha$  at tyrosine 42, leading to dissociation of the I $\kappa$ B $\alpha$ :NF- $\kappa$ B complex without degradation of I $\kappa$ B $\alpha$ .<sup>21</sup> The third pathway involves enhanced processing of p100 upon phosphorylation by IKK $\alpha$ . Phosphorylation triggers processing of p100 to p52 within the p100:relB complex, which results in unmasking of nuclear localization signal of p52:relB heterodimer.<sup>22</sup> NF- $\kappa$ B then moves into the nucleus and binds to response elements in the promoter region of its target genes and activates transcription. NF- $\kappa$ B activity in the nucleus is further regulated by phosphorylation, acetylation, and coactivator and corepressor interactions.<sup>20,23,24</sup> NF- $\kappa$ B terminates expression of its target genes by inducing I $\kappa$ B $\alpha$  expression, and the newly synthesized I $\kappa$ B $\alpha$  sequesters NF- $\kappa$ B back into the cytoplasm.<sup>25</sup> Thus, several regulatory steps including controlled subcellular localization, heterodimerization among different NF- $\kappa$ B family members, and a negative feedback loop control tissue-specific and inducible gene expression by NF- $\kappa$ B. Any aberration in these steps can lead to uncontrolled gene expression by NF- $\kappa$ B, with profound impact on cell growth, differentiation, apoptosis, and immune response.

## NF- $\kappa$ B Target Genes

NF- $\kappa$ B regulates expression of genes involved in diverse functions: a partial list of genes that have potential impact on cancer is shown in Table 1. These genes control cell proliferation (cyclin D1, c-Myc), cell adhesion

TABLE 1. NF- $\kappa$ B-Regulated genes relevant for cancer

Genes	Function
NF- $\kappa$ B-inducible genes	
Cyclin D1	Proliferation
c-Myc	Proliferation
ICAM-1	Cell adhesion
VCAM-1	Cell adhesion
ELAM-1	Cell adhesion
Integrin $\alpha$ V	Cell adhesion, proliferation, metastasis
TNF $\alpha$	Angiogenesis, cachexia, osteoclast activation
IL-1	Angiogenesis, cachexia, osteoclast activation
IL-6	Migration, angiogenesis, metastasis, cachexia
IL-8	Angiogenesis, cachexia
iNOS	Proliferation, angiogenesis, metastasis
COX-2	Proliferation, angiogenesis, metastasis
MIP-1, MIP-2	Migration, angiogenesis, osteoclast activation
M-CSF	Osteoclast activation
VEGF	Angiogenesis
Gro-1	Metastasis, angiogenesis
UPA	Invasion, migration, metastasis
MMP9	Metastasis
Haparanase	Metastasis
Bcl-2	Antiapoptosis, chemoresistance
Bcl-XL	Antiapoptosis, chemoresistance
AL/Bfl-1	Antiapoptosis
XIAP	Antiapoptosis
clAP-1, clAP-2	Antiapoptosis
TRAF-1, TRAF-2	Antiapoptosis
cFLIP	Antiapoptosis
Mn-SOD	Antiapoptosis, chemoresistance
Glutathione synthase	Antiapoptosis, chemoresistance
GADD45 $\beta$	Antiapoptosis
AKT1	Antiapoptosis, chemoresistance
NF- $\kappa$ B-repressible genes	
GADD153/CHOP	Proapoptosis
TIMP1, TIMP2	Metastasis suppressor
Plasminogen activator inhibitor	Metastasis suppressor

(ICAM-1, VCAM-1, ELAM1, Integrin  $\alpha$ V), immune response (TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-6, IL-8, chemokines such as MIP $\alpha$ , MCP-1), cell migration (urokinase plasminogen activator [uPA], matrix metalloproteinase 9), angiogenesis (VEGF) and antiapoptosis (Bcl-2, Bcl-X<sub>L</sub>, A1/Bfl1, A20, XIAP, clAP-1, clAP-2, TRAF-1, TRAF-2, cFLIP, manganese superoxide dismutase,  $\gamma$ -glutathione synthase, and GADD45 $\beta$ ).<sup>16,26-40</sup> Several of these genes are involved in the normal developmental processes. Consistent with the role of NF- $\kappa$ B in embryonic development, "knocking-out" of



the *RelA* subunit of NF- $\kappa$ B leads to embryonic lethality.<sup>41</sup> Similar defects were also observed in mice lacking IKK activity.<sup>42,43</sup> Knockout mice lacking NF- $\kappa$ B1, NF- $\kappa$ B2, and *c-Rel* develop normally but show defects in the immune response.<sup>44-47</sup> Osteopetrosis develops in mice lacking both NF- $\kappa$ B1 and NF- $\kappa$ B2, suggesting a role for NF- $\kappa$ B-regulated genes in osteoclast activation.<sup>48</sup>

## NF- $\kappa$ B in Mammary Gland Development

Development of the mammary gland is a complex process involving extensive proliferation of mammary epithelial cells during pregnancy, differentiation during lactation, and extensive apoptosis of the secretory epithelial cells during involution.<sup>49,50</sup> Mouse and rat models have been used to determine the potential role of NF- $\kappa$ B in these processes. NF- $\kappa$ B DNA binding activity is elevated during pregnancy, suppressed during lactation, and reactivated during involution.<sup>51,52</sup> However, reactivation of NF- $\kappa$ B during involution occurs exclusively in nonapoptotic epithelial cells, which suggests a prosurvival function of NF- $\kappa$ B during involution.<sup>53</sup> The identity of subunits of NF- $\kappa$ B activated in the normal mammary gland remains controversial, as the p65:p50 heterodimer and the p50 homodimer have both been reported as activated by different groups.<sup>53,54</sup> Nonetheless, the importance of NF- $\kappa$ B in proliferation and branching of mammary epithelial cells has been confirmed by transplanting IkB $\alpha$ -deficient mammary epithelium into the mammary fat pad of wild-type mice.<sup>55</sup> IkB $\alpha$ -deficient cells, in which NF- $\kappa$ B is constitutively localized in the nucleus, showed increased proliferation, lateral ductal branching, and pervasive intraductal hyperplasia compared with wild-type counterparts. Furthermore, the extracellular matrix was reduced adjacent to IkB $\alpha$ -deficient epithelium. Thus, it appears that NF- $\kappa$ B is required for proliferation of mammary epithelial cells, blocking of terminal differentiation, and/or delaying the onset of apoptosis.

TNF $\alpha$  and RANKL are the major physiologic activators of NF- $\kappa$ B in the mammary gland.<sup>56,57</sup> A systematic genetic analysis in mice revealed that activation of NF- $\kappa$ B and the resulting proliferation of mammary epithelial cells during pregnancy occurs through a linear cascade: RANKL  $\rightarrow$  RANK  $\rightarrow$  IKK $\alpha$   $\rightarrow$  IkB $\alpha$   $\rightarrow$  NF- $\kappa$ B  $\rightarrow$  cyclin D1.<sup>57</sup> Deletion or functional inactivation of RANKL, RANK, IKK $\alpha$ , or cyclin D1 in mice results in similar defects in proliferation of mammary epithelial cells.<sup>57,60</sup> With respect to tumorigenesis, any genetic mutation that leads to aberrant activation of this cascade could lead to increased proliferation and predispose mammary epithelial cells for transformation.

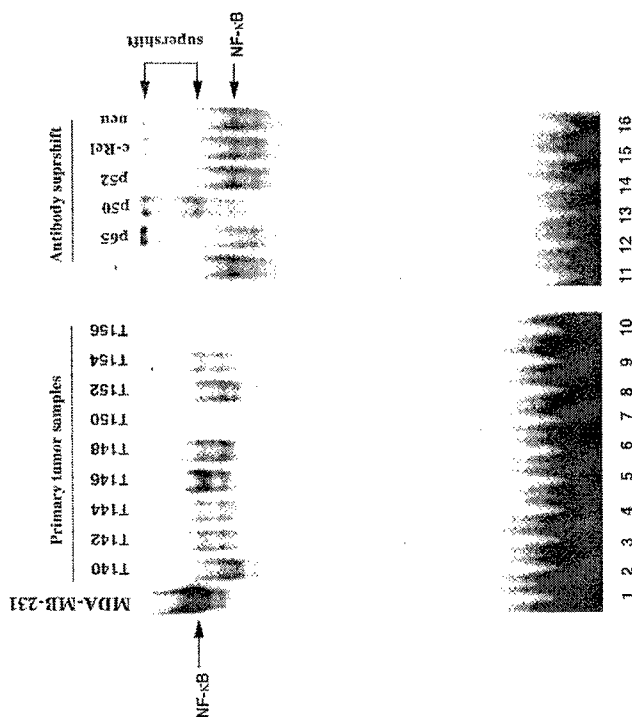


Fig 2. NF- $\kappa$ B DNA binding activity in primary breast cancer. NF- $\kappa$ B DNA binding activity was measured by electrophoretic mobility shift assay. MDA-MB-231 cell extract was used as positive control. NF- $\kappa$ B DNA complex contains p65 and p50 subunits as determined by antibody supershift assay [lanes 11 to 16].

## Constitutive Activation of NF- $\kappa$ B in Breast Cancer

Progression of breast cancer from a benign to a malignant phenotype is accompanied by overexpression of several growth factors, cytokines, and chemokines by cancer cells. Some of these growth factors and cytokines can induce the expression of prometastatic genes through NF- $\kappa$ B. One example is uPA, whose overexpression correlates with poor clinical outcome.<sup>61</sup> These observations prompted our laboratory to investigate whether NF- $\kappa$ B is constitutively active in breast cancer. NF- $\kappa$ B DNA binding activity was examined in a panel of established breast cancer cell lines. In general, elevated NF- $\kappa$ B DNA binding activity was observed in breast cancer cell lines with invasive and metastatic growth properties.<sup>62</sup> Furthermore, extracts from primary breast tumors showed elevated NF- $\kappa$ B DNA binding activity (Fig 2). Increased expression of IL-6 and uPA was observed in breast cancer cell lines with constitutive NF- $\kappa$ B DNA binding activity (Fig 3).<sup>63</sup> Sovak et



30% of breast cancers are ER $\alpha$ -negative at the time of initial diagnosis, and the 5-year survival rate for these patients is <50%.<sup>4</sup> We propose NF- $\kappa$ B as the molecular target for ER $\alpha$ -negative breast cancers.

## Mechanisms of Constitutive NF- $\kappa$ B Activation

We and others have shown the involvement of EGFR, IL-1 $\alpha$ , and heregulin in constitutive activation of NF- $\kappa$ B in breast cancer cells.<sup>69-73</sup> Biswas et al.<sup>72</sup> showed a correlation between EGFR overexpression and increased NF- $\kappa$ B activity in ER $\alpha$ -negative breast cancers. Inhibitors of EGFR reduced NF- $\kappa$ B activity and cell growth. We observed that a subset of ER $\alpha$ -negative breast cancer cells secrete IL-1 $\alpha$ , which activates NF- $\kappa$ B in both cancer and stromal cells. Neutralizing antibodies against IL-1 $\alpha$  blocked NF- $\kappa$ B activity and reduced NF- $\kappa$ B-regulated gene expression in both cancer and stromal cells.<sup>73</sup> To identify the specific oncogenes that may be involved in NF- $\kappa$ B activation, we recently used mammary tumor cell lines derived from transgenic mice that specifically overexpress oncogenes in their mammary gland. Constitutive NF- $\kappa$ B DNA binding activity was observed in cell lines from heregulin transgenic mice but not from *c-Myc*, *ras*, or *her2/neu* transgenic mice.<sup>70</sup> However, *her2/neu* potentiated TNF-induced NF- $\kappa$ B activation and stimulated cell growth in the presence of TNF. Interestingly, overexpression of EGFR, heregulin, and *her2/neu* is usually observed in ER $\alpha$ -negative breast cancers and is associated with poor prognosis.<sup>74,75</sup> Thus, it is likely that NF- $\kappa$ B is the downstream target of signal transduction pathways that are responsible for growth of ER $\alpha$ -negative breast cancers.

Apart from EGFR, IL-1 $\alpha$ , heregulin, and *her2/neu*, breast cancers express bone morphogenic factor 4, PDGF, and transforming growth factor- $\alpha$ .<sup>76-79</sup> These growth factors may be involved in autocrine and paracrine induction of NF- $\kappa$ B in the tumor microenvironment. However, their contribution in constitutive NF- $\kappa$ B activation in breast cancer is yet to be verified. In addition to production of growth factors and cytokines, hypoxic and acidic pH conditions are common in the tumor microenvironment, and both of these conditions may contribute to constitutive NF- $\kappa$ B activation in breast cancer.<sup>80</sup> Furthermore, elevated expression and/or activity of IKK and casein kinase II is linked to constitutive NF- $\kappa$ B activation in breast cancer.<sup>81</sup>

## NF- $\kappa$ B Target Genes in Breast Cancer

Although more than 180 genes are induced by NF- $\kappa$ B, not all of them are expressed in breast cancer. To identify NF- $\kappa$ B-regulated genes in breast cancer cells, we created MDA-MB-231 breast cancer cells over-

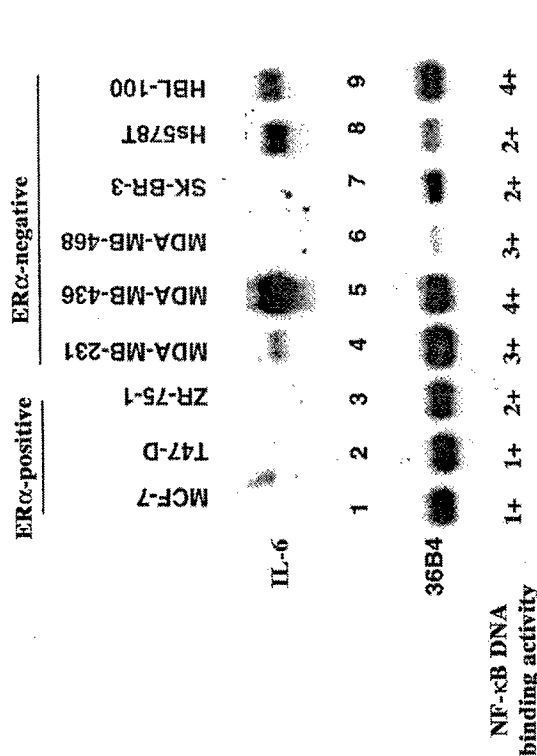


Fig 3. Breast cancer cells with constitutive NF- $\kappa$ B overexpress urokinase plasminogen activator. Urokinase plasminogen activator expression was measured by Northern blotting. Ribosomal protein gene 36B4 was used as control.

al<sup>64</sup> reported constitutive NF- $\kappa$ B DNA binding activity in 65% of primary breast cancers. The NF- $\kappa$ B:DNA complex that we observed in cell lines as well as primary breast cancers was predominantly a heterodimer of p65:p50 (Fig 2). Constitutive nuclear localization of p50, p52, c-*Rel*, and Bcl-3 and overexpression of p100/p52 in breast cancer have been reported by others.<sup>65,66</sup>

It was apparent in our studies that the majority of breast cancer cells with constitutive NF- $\kappa$ B DNA binding and transcriptional activity are estrogen receptor- $\alpha$  (ER $\alpha$ )-negative.<sup>62</sup> Because the ER $\alpha$ -negative phenotype correlates with poor prognosis, we explored the possibility that ER $\alpha$  negatively regulates NF- $\kappa$ B activity in breast cancer cells. Indeed, although DNA binding of NF- $\kappa$ B is inducible in ER $\alpha$ -positive breast cancer cells, NF- $\kappa$ B is transcriptionally less competent in these cells.<sup>62</sup> ER $\alpha$  physically associates with DNA-bound NF- $\kappa$ B and interferes with its transactivation.<sup>67</sup> This function of ER $\alpha$ , which is called transrepression, is essential to prevent invasion by cancer cells.<sup>68</sup> This may explain the differences in the metastatic potential of ER $\alpha$ -positive and ER $\alpha$ -negative breast cancers.<sup>3</sup> Thus, it appears that constitutive activation of NF- $\kappa$ B is more relevant to ER $\alpha$ -negative breast cancers. Approximately



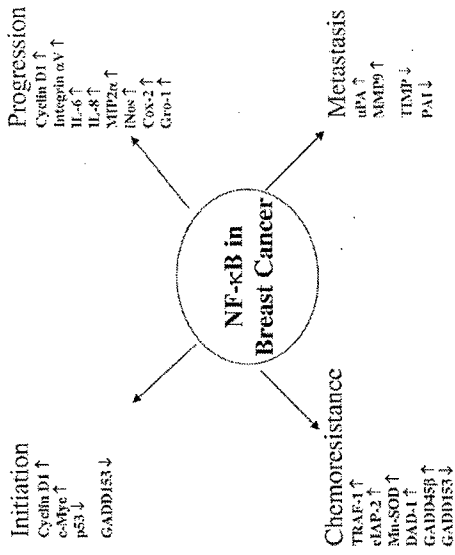


Fig 4. Potential contributions of NF- $\kappa$ B in breast cancer initiation, progression, metastasis, and chemoresistance. NF- $\kappa$ B-regulated genes involved in breast cancer are shown.

expressing I $\kappa$ B $\alpha$ .<sup>82</sup> MDA-MB-231 cells contain constitutively active NF- $\kappa$ B because of IL-1 $\alpha$  expression and EGFR overexpression.<sup>62,69</sup>

Comparative analysis of parental and I $\kappa$ B $\alpha$ -overexpressing cells by cDNA microarray and RNase protection assay identified several NF- $\kappa$ B-regulated genes. All but one of them are induced by NF- $\kappa$ B. Genes induced by NF- $\kappa$ B include uPA, IL-6, IL-8, macrophage inhibitory protein-2- $\alpha$  (MIP2 $\alpha$ ), defender against cell death (DAD), ICAM-1, glutathione S-transferase M4, Cu/Zn superoxide dismutase 1, c-IAP2, TRAF-1, and Mn-SOD.<sup>63,82</sup> NF- $\kappa$ B-inducible genes in breast cancer identified by microarray analysis by others include matrix metalloproteinase (MMP)9, VEGF, cyclin D1, and *Gro-1*.<sup>3,4,83,84</sup> We have also observed repression of GADD153/CHOP by NF- $\kappa$ B in breast cancer cells.<sup>85</sup> We expect further additions to the list of genes induced by NF- $\kappa$ B in the near future. The potential role of these genes in breast cancer initiation, progression, metastasis, and chemoresistance is shown diagrammatically in Fig 4.

## NF- $\kappa$ B in Breast Cancer Initiation, Progression, and Metastasis

### Initiation

DMBA- or benzo[a]pyrene-induced mammary tumors in rats and in vitro transformation assay of human mammary epithelial cells obtained

from reduction mammaplasty have been used to analyze NF- $\kappa$ B activation during initiation of breast cancer.<sup>86</sup> With both assays, activation of the p50:p65 heterodimer was observed before malignant transformation.<sup>86</sup> The c-Myc oncogene, which is overexpressed in 30% of breast cancers, appears to be the target of NF- $\kappa$ B in this system.<sup>86</sup> NF- $\kappa$ B may directly increase cyclin D1 expression, whereas c-Myc may increase the expression of cyclin A and cyclin E, leading to rapid cell proliferation.<sup>26,27,87</sup> Cyclin D1 is overexpressed at the early stages of breast cancer progression, such as ductal carcinoma in situ but not in premalignant lesions (ie, LCFS).<sup>88,89</sup> Cyclin D1 overexpression in ER $\alpha$ -negative but not ER $\alpha$ -positive breast cancers correlates with poor prognosis.<sup>90</sup> Furthermore, cyclin D1 is absolutely necessary for her2/neu and *ras* oncogene-induced mammary tumor formation.<sup>91</sup> Thus, growth factor/oncogenic pathways activated early in premalignant cells (particularly ER $\alpha$ -negative) may utilize NF- $\kappa$ B to induce cyclin D1 expression and transformation.

Modulation of p53 activity may be another mechanism by which NF- $\kappa$ B promotes breast cancer initiation. p53 is a tumor suppressor gene that functions as a gatekeeper by arresting cells with damaged DNA at the G0/G1 phase of the cell cycle. If DNA damage is less extensive, it is repaired and cells progress through the cell cycle. If DNA damage is extensive, p53 triggers apoptosis.<sup>92</sup> Several recent publications, with one exception, indicate that NF- $\kappa$ B inhibits p53 activity.<sup>93-95</sup> Thus, by reducing p53 activity, NF- $\kappa$ B may enhance survival of cells with damaged DNA. Surviving cells with damaged DNA accumulate additional mutations, leading to transformation. Our recent studies indicate that NF- $\kappa$ B represses expression of the GADD153/CHOP gene.<sup>85</sup> GADD153/CHOP is usually induced in cells exposed to environmental toxins and during stress of endoplasmic reticulum seen with several diseases.<sup>96</sup> Depending on the severity of DNA damage and stress, GADD153 triggers apoptosis. By reducing GADD153 expression, NF- $\kappa$ B may promote survival and transformation of cells with damaged DNA.

### Progression

Progression of cancer requires proliferation, angiogenesis, and protection against apoptotic signals originating from the immune system. Several of the NF- $\kappa$ B-regulated genes can aid in this process. For example, NF- $\kappa$ B can increase cell proliferation through c-Myc and cyclin D1. NF- $\kappa$ B can promote angiogenesis by inducing the expression of IL-6, IL-8, *Gro-1*, and VEGF.<sup>9,16,97-99</sup> NF- $\kappa$ B also affects angiogenesis indirectly through stromal cells. We have observed that IL-1 $\alpha$  secreted by



breast cancer cells induces NF- $\kappa$ B in stromal fibroblasts, resulting in increased expression of IL-6 and IL-8 by these cells.<sup>69,73</sup> IL-6, although not directly linked to angiogenesis, can induce the expression of tissue factor 1 in stromal cells.<sup>100</sup> Tissue factor 1 is a potent inducer of angiogenesis.<sup>101</sup> Finally, NF- $\kappa$ B-regulated genes, including Mn-SOD, cIAP-2, DAD-1, and TRAF-1, can protect cancer cells from the cytotoxic effects of TNF.<sup>35,102,103</sup> It was reported recently that normal mammary epithelial cells secrete Fas ligand, which triggers apoptosis of surrounding cancer cells.<sup>104</sup> However, cancer cells can protect themselves against Fas ligand through activation of NF- $\kappa$ B.<sup>104</sup>

The serine/threonine kinase AKT functions as a major survival protein for a variety of cell types.<sup>105</sup> Overexpression of this kinase alone is sufficient to induce mammary gland hyperplasia.<sup>106</sup> It was demonstrated recently that AKT is one of the target genes of NF- $\kappa$ B.<sup>107</sup> Thus, constitutive NF- $\kappa$ B activation may lead to elevated AKT, resulting in prolonged survival of immortalized and/or transformed cells in the mammary gland. Consistent with this possibility, AKT is overexpressed in breast cancer.<sup>108,109</sup>

Additional candidate NF- $\kappa$ B-regulated genes involved in breast cancer progression include inducible nitric oxide synthase and COX-2, both of which are overexpressed in advanced breast cancers.<sup>110,111</sup> Overexpression of COX-2 and inducible nitric oxide synthase correlates with increased angiogenesis and metastatic disease.<sup>110,112</sup> COX-2 overexpression, which is observed in ~40% of breast cancers, is associated with the ER $\alpha$ -negative phenotype and unfavorable prognosis.<sup>113</sup>

## Metastasis

The metastasis of malignant cells is a multistep process involving (1) extracellular matrix degradation and subsequent detachment of cancer cells from the primary tumor; (2) invasion of surrounding tissue; (3) penetration of blood and/or lymphatic vessels (also called intravasation); (4) survival in the blood circulation, transport to new sites, and arrest of cancer cells in the microcirculation; (5) migration of cancer cells through the vessel wall into the interstitial space (extravasation); and (6) proliferation of cancer cells in the target organs (colonization).<sup>114</sup> Two mutually dependent protease systems are required for extracellular matrix degradation.<sup>115</sup> The first system includes MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13. These proteins are secreted as pro-MMPs, and most of them are activated by the second protease system. The second system involves the uPA/urokinase plasminogen activator receptor (uPAR)/plasminogen system. The uPA/uPAR converts plasminogen to

plasmin, which activates MMPs.<sup>115</sup> The uPA/uPAR and MMP-9 are essential for intravasation of a number of cancer cell lines, including MDA-MB-231 breast cancer cells.<sup>116</sup> Although overexpression of uPA/uPAR/MMP-9 is not necessary for survival and attachment of cancer cells to the microvasculature, overexpression of uPA/uPAR is essential for migration and colonization at the target site.<sup>116-118</sup> NF- $\kappa$ B can promote extracellular matrix degradation, migration, and colonization at the target site by directly inducing the expression of uPA and MMP-9, thereby increasing metastasis.<sup>33,34,119</sup> NF- $\kappa$ B can also increase MMP-2 indirectly by synergizing with the AP-1 transcription factor.<sup>67</sup> NF- $\kappa$ B has also been shown to increase the expression of prometastatic heparanase and decrease the expression of antimetastatic tissue inhibitors of MMP-1 and MMP-2 and plasminogen activator inhibitor 2.<sup>120</sup> Consistent with the role of NF- $\kappa$ B in the degradation of extracellular matrix, mammary glands of mice transplanted with I $\kappa$ B $\alpha$ -deficient mammary epithelial cells showed a lower level of intact extracellular matrix.<sup>55</sup>

## NF- $\kappa$ B in Osteolytic Bone Lesions and Cachexia

While cachexia contributes to mortality in patients, osteolytic lesions do not. Bone metastasis develops in ~70% of patients with breast cancer, whereas cachexia develops in 30% of patients.<sup>121,122</sup> Several factors, including IL-1, IL-6, RANKL, parathyroid hormone-related protein (PTHrP), MIP1 $\alpha$ , and macrophage colony stimulating factor (M-CSF), have been implicated in osteoclast activation.<sup>123,124</sup> Cancer cells secrete most of these factors, and the expression of some of these factors (IL-1, IL-6, MIP1 $\alpha$ , and M-CSF) is dependent on NF- $\kappa$ B. In fact, MDA-MB-231 breast cancer cells, which have been used extensively for studying osteolytic bone metastasis in a xenograft model, express IL-1, IL-6, PTHrP, and M-CSF.<sup>125,126</sup> We have observed that expression of IL-1 and IL-6 in these cells is dependent on NF- $\kappa$ B. Thus, extensive bone loss can be anticipated when breast cancer cells, with constitutively active NF- $\kappa$ B, increase the level of osteoclastogenic factors in the bone microenvironment after metastasis.

IL-6, IL-8, TNF $\alpha$ , interferon- $\gamma$  (IFN $\gamma$ ), leukemia inhibitory factor, myostatin/GDF8, protein mobilizing factor (PMF), and lipid mobilizing factor (LMF) are some of the factors involved in cachexia.<sup>127-129</sup> IFN $\gamma$ , in combination with TNF $\alpha$ , has been shown to induce cachexia through NF- $\kappa$ B-dependent destabilization of MyoD mRNA in muscle.<sup>130</sup> Because expression of IL-6, IL-8, TNF $\alpha$ , and IFN $\gamma$  is dependent on NF- $\kappa$ B, it is possible that breast cancers with constitutively active NF- $\kappa$ B overexpress these cachexia-inducing factors.



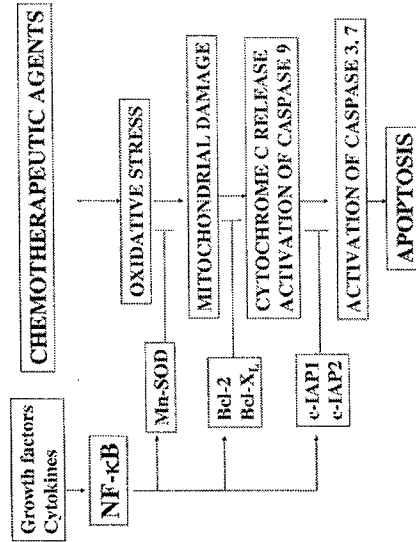


Fig 5. Model depicting mechanism of chemoresistance by NF- $\kappa$ B.

## NF- $\kappa$ B-Mediated Chemoresistance

It was recognized a decade ago that NF- $\kappa$ B is activated in cancer cells after exposure to chemotherapeutic agents including taxol, doxorubicin, daunorubicin, and camptothecin.<sup>131-133</sup> These initial results provided a link between cell death and NF- $\kappa$ B activation. However, a definitive role for NF- $\kappa$ B in cell survival, with or without exposure to chemotherapy, was not established until 1996. Cells lacking NF- $\kappa$ B activity, obtained either through gene knockout or through overexpression of I $\kappa$ B $\alpha$  super-repressor (a mutant form of I $\kappa$ B $\alpha$  in which S32 and S36 are mutated to be nonphosphorylatable alanine and hence nondegradable), were found to be more sensitive to daunorubicin and TNF.<sup>134-136</sup> Subsequent studies have shown that NF- $\kappa$ B is essential for the expression of several antiapoptotic genes including *Bcl-2*, *Bcl-X<sub>L</sub>*, TRAF-1, TRAF-2, Bfl1/A1, cFlip, XIAP, cIAP-1, cIAP-2, and Mn-SOD.<sup>16,17,35</sup> A schematic view of how these proteins may confer resistance to chemotherapy is shown in Fig 5. Chemotherapeutic drugs usually cause oxidative stress, leading to mitochondrial damage. Cytochrome C is released from damaged mitochondria, which activates the apoptosome. The apoptosome cleaves procaspase 9, releasing active caspase 9.<sup>137-139</sup> Caspase 9 then cleaves procaspase 3 to caspase 3, which is an executioner caspase. Caspase 3 activates specific DNases involved in cleavage of chromosomal DNA. Caspase 3 cleaves several important proteins, including PARP. Mn-SOD confers resistance to chemotherapy by reducing oxidative stress. Indeed, overexpression of Mn-SOD alone in breast cancer cells is sufficient for

resistance against taxol, H<sub>2</sub>O<sub>2</sub>, and okadaic acid.<sup>102</sup> *Bcl-2* and *Bcl-X<sub>L</sub>* confer resistance to chemotherapy by preventing the release of cytochrome C from the mitochondria. XIAP, cIAP-1, and cIAP-2 reduce cell death by preventing activation of caspase 9 as well as by inhibiting the activity of caspase 3.<sup>138</sup> Thus, NF- $\kappa$ B-regulated genes confer resistance to chemotherapy by interfering at multiple sites in the normal pathway of cell death.

To understand the role of NF- $\kappa$ B in resistance to chemotherapy, we generated MDA-MB-231 cells overexpressing I $\kappa$ B $\alpha$ SR.<sup>82</sup> The expression of antiapoptotic genes TRAF-1, cIAP-2, DAD-1, and Mn-SOD was lower in I $\kappa$ B $\alpha$ SR cells compared with parental cells. We then examined the sensitivity of parental and I $\kappa$ B $\alpha$ SR cells to taxol. I $\kappa$ B $\alpha$ SR cells were more sensitive to taxol-induced G2/M arrest and apoptosis.<sup>82</sup> Thus, NF- $\kappa$ B-regulated genes appear to control the response of breast cancer cells to taxol.

Gene therapy with I $\kappa$ B $\alpha$ SR is one mechanism of overcoming chemoresistance. In fact, adenovirus-mediated gene therapy with I $\kappa$ B $\alpha$ SR has been shown to increase sensitivity of colorectal carcinoma cells to CPT11 in a xenograft model.<sup>140</sup> To avoid the difficulty in targeting I $\kappa$ B $\alpha$ SR to breast cancers, we evaluated the utility of various exogenous chemical inhibitors of NF- $\kappa$ B as chemosensitizers to taxol. Parthenolide, an NF- $\kappa$ B inhibitor derived from the herb feverfew,<sup>141</sup> increased the sensitivity of MDA-MB-231 and HBL-100 cells to taxol.<sup>82</sup> Weldon et al<sup>142</sup> made similar observations. Pharmacologic inhibition of NF- $\kappa$ B with BAY 11-7082 increased sensitivity of MCF-7 APO+ breast cancer cells to doxorubicin and taxol. Although there is one contradicting report suggesting the requirement of NF- $\kappa$ B for chemotherapy-induced death of breast cancer cells,<sup>143</sup> most of the current literature favors the antiapoptotic function of NF- $\kappa$ B in breast cancer and points to a new therapeutic direction.

## Inhibitors of NF- $\kappa$ B as Chemosensitizers of Breast Cancer

More than 100 compounds with anti-NF- $\kappa$ B activity have been described in the literature.<sup>15</sup> These include folk medicines derived from natural sources, known for their anti-inflammatory properties, which have been in clinical use for many years. It was reported in 1995 that the anti-inflammatory action of glucocorticoids was due to inhibition of NF- $\kappa$ B activity.<sup>144,145</sup> Subsequently, a number of natural and synthetic compounds with anti-inflammatory properties have been tested for anti-NF- $\kappa$ B activity, and the anti-inflammatory medicinal plants from the



genera *Barberris*, *Coptis*, and *Gravel Root* are receiving renewed attention.<sup>146,147</sup> Below, we describe some of the compounds, which may be explored as chemosensitizers for breast cancer.

### *Parthenolide*

Parthenolide is the active ingredient of the herb feverfew.<sup>141</sup> Feverfew is being used as a migraine prophylaxis.<sup>148</sup> It inhibits NF- $\kappa$ B activation by reducing IKK activity.<sup>149</sup> Parthenolide also inhibits DNA binding of the STAT family of transcription factors, which probably is an indirect result of NF- $\kappa$ B inhibition.<sup>150</sup> At a higher concentration, parthenolide inhibits MAP kinase activity.<sup>151</sup> After obtaining encouraging *in vitro* results, we are exploring the combination of parthenolide and taxanes in a xenograft model of breast cancer. In our nude mice studies, we did not observe toxic side effects of prolonged parthenolide treatment. One limitation of parthenolide is its relatively short half-life. It inhibited NF- $\kappa$ B DNA binding activity for only 5 hours.<sup>85</sup> It may therefore be necessary to develop more stable analogues. Nonetheless, we remain optimistic that our ongoing studies will reveal a useful role for parthenolide as a chemosensitizer.

### *Antioxidants*

Antioxidants with NF- $\kappa$ B-inhibitory properties include curcumin and epigallocatechin-3-gallate (EGCG).<sup>152,153</sup> Curcumin is a common ingredient in Asian cooking and has been tested in animal models as a chemoprevention agent.<sup>154,155</sup> EGCG is the active ingredient of green tea and is currently being tested as a chemoprevention agent also.<sup>153</sup> Although these two compounds are promising, their use as chemosensitizers may be limited because relatively higher concentrations (30 to 60  $\mu$ mol/L) are necessary to achieve NF- $\kappa$ B inhibition.

### *IKK Inhibitors*

Aspirin, ibuprofen, 4-hydroxy-2-nonenal, and sanguinarine are potent inhibitors of IKK.<sup>156,160</sup> It will be interesting to see whether prior treatment with aspirin or ibuprofen increases sensitivity of breast cancer cells to chemotherapy. Recently, a peptide inhibitor of IKK activity has been described and may function as a chemosensitizer.<sup>161</sup> The mechanism of NF- $\kappa$ B inhibition by several compounds is not known, and it is presumed that some of these inhibitors target IKK. IKK inhibitors hold more promise than other broad-spectrum inhibitors of NF- $\kappa$ B because of fewer undesirable side effects.

### *Proteasome Inhibitors*

Inhibitors of the 26S proteasome (a cellular factory involved in controlled degradation of proteins), which block degradation of phosphorylated I $\kappa$ B $\alpha$ , have been used as NF- $\kappa$ B inhibitors for *in vitro* studies. These compounds include MG132, lactacystine, ALLnL, and cyclosporine A.<sup>15</sup> PS341, which is in phase II and phase III clinical trials, is a potent inhibitor of NF- $\kappa$ B.<sup>162</sup> The major molecular target of PS341 in melanoma is NF- $\kappa$ B, as constitutive activation of NF- $\kappa$ B is frequently observed in this cancer.<sup>163</sup> We believe that PS341 will have similar effects in breast cancer either as a single agent or in combination with chemotherapy.

### *Glucocorticoids*

The anti-inflammatory activity of glucocorticoids is largely due to inhibition of NF- $\kappa$ B.<sup>144,145</sup> Inhibition of NF- $\kappa$ B activity by glucocorticoids involves two independent cell type-specific mechanisms. In certain cell types, glucocorticoids directly induce the expression of I $\kappa$ B $\alpha$ , thereby reducing nuclear translocation and DNA binding of NF- $\kappa$ B.<sup>144,145</sup> In most cell types, however, glucocorticoids inhibit NF- $\kappa$ B activity through transcriptional interference.<sup>164,165</sup> The glucocorticoid receptor, upon binding of glucocorticoids, translocates to the nucleus, where it interacts with the p65 subunit of NF- $\kappa$ B. The p65:glucocorticoid receptor complex is transcriptionally inactive. Elegant knock-in mutation studies have proved that this function of the glucocorticoid receptor is important for repression of the inflammatory response.<sup>166</sup> Some of the chemotherapy combinations include glucocorticoids mainly to overcome treatment side effects.<sup>167,168</sup> It is possible that glucocorticoids enhance sensitivity of cancer cells to chemotherapy by reducing NF- $\kappa$ B-dependent expression of antiapoptotic genes.

### *PPAR $\gamma$ Agonists*

Peroxisome proliferator activator receptor- $\gamma$  (PPAR $\gamma$ ) agonists such as troglitazone are potent antidiabetic drugs in clinical use.<sup>169</sup> PPAR $\gamma$  agonists are also currently being tested as chemoprevention agents.<sup>170,171</sup> These agonists can inhibit NF- $\kappa$ B activity by at least two mechanisms. PPAR $\gamma$  directly interacts with the p65 subunit of NF- $\kappa$ B and inhibits its transactivation function.<sup>172,173</sup> PPAR $\gamma$  agonists also induce the expression of the tumor suppressor gene PTEN.<sup>174</sup> PTEN inhibits the activity of AKT, a kinase that increases NF- $\kappa$ B DNA binding as well as transactivation.<sup>175,176</sup> Because PPAR $\gamma$  is expressed in breast cancer cells, its agonists may function as chemosensitizers in breast cancer by reducing



the expression of NF- $\kappa$ B-regulated genes.<sup>177</sup> Consistent with this possibility, the natural PPAR- $\gamma$  agonist 15-deoxy- $\delta^{13,15}$ -prostaglandin J2 inhibits expression of the NF- $\kappa$ B-inducible genes MCP-1 and GADD45 $\beta$  and increases expression of the NF- $\kappa$ B-repressible gene CHOP/GADD153 in MDA-MB-231 cells.<sup>177</sup>

### *Antisense Oligonucleotides*

Antisense oligonucleotides against p65 have been used in the nude mice model to evaluate the role of NF- $\kappa$ B in tumorigenicity and experimental colitis. The antisense oligonucleotides reduced *in vitro* growth and tumor formation by breast cancer cells, osteosarcoma, melanoma, and colon carcinoma cells.<sup>178</sup> Similarly, antisense oligonucleotides also reduced experimental colitis.<sup>179</sup> Recently developed RNA interference (RNAi) technology is more promising than the antisense technique for functional inactivation of a gene of interest.<sup>180</sup> RNAi is a short, 21-base-long, double-stranded RNA molecule corresponding to a gene of interest, which through an unknown mechanism triggers degradation of the specific RNA. We anticipate that this technique will become a more powerful tool in preclinical and clinical studies for inactivation of NF- $\kappa$ B.

### *Potential Complications of Long-Term NF- $\kappa$ B Inhibition*

The importance of NF- $\kappa$ B in the immune system led to initial concerns in developing NF- $\kappa$ B inhibitors as therapeutic agents. This concern was further strengthened by knockout studies showing an important role of NF- $\kappa$ B in liver organogenesis.<sup>41</sup> Embryonic death caused by massive hepatic apoptosis was observed in p65 knockout mice. On the basis of these results, hepatotoxicity was predicted with NF- $\kappa$ B inhibitors. However, p65 does not appear to be required for the survival of adult hepatocytes because conditional knockout of p65 in adult mice is not deleterious, except for a modest increase in bacterial infection.<sup>181</sup> The common use of drugs such as aspirin and glucocorticoids, which inhibit NF- $\kappa$ B activity, has lessened concern over the therapeutic use of NF- $\kappa$ B inhibitors, but much work remains.

### *NF- $\kappa$ B in Anti-Estrogen Resistance*

Approximately 50% of breast cancers are ER $\alpha$ -positive at the time of diagnosis. Although most patients respond to anti-estrogen therapy, *in vitro* studies have demonstrated an eventual resistance to therapy.<sup>182</sup> Dr Robert Clarke's group has recently used a xenograft model to study the mechanism of resistance to pure anti-estrogen, ICI182,780.<sup>183</sup> Resistance to ICI182,780 was accompanied by increased NF- $\kappa$ B activity. These

ICI-resistant cells were growth-inhibited by parthenolide, suggesting the requirement of constitutive NF- $\kappa$ B for the survival of these cells. The mechanisms for constitutive NF- $\kappa$ B activation in these cells remains to be determined. It is possible that the ER $\alpha$  lost its ability to transrepress NF- $\kappa$ B activity because of posttranslation modification or altered stability.

### *Summary and Future Directions*

Molecular target-based therapy is becoming a reality with the completion of the human genome project and the development of microarray technology. Hereceptin was the first successful molecular target-based therapy developed for breast cancer. Unfortunately, it is beneficial in only 30% of patients with breast cancer. Because NF- $\kappa$ B is downstream of a number of growth factor/oncogene-activated signal transduction pathways, particularly in ER $\alpha$ -negative breast cancers, it is an ideal target for intervention. In this article, we have highlighted how NF- $\kappa$ B controls breast cancer initiation, progression, metastasis, and resistance to chemotherapy. A number of NF- $\kappa$ B inhibitors derived from natural products have already been characterized. These NF- $\kappa$ B inhibitors provide a perfect opportunity for the rational design of clinical trials that combine alternative medicine with conventional medicine. However, several challenges still exist, including the identification of compounds that inhibit NF- $\kappa$ B at nanomolar concentrations, compounds that inhibit NF- $\kappa$ B for a longer duration, production of orally active compounds, and determining the ideal sequence and duration of administration. We believe that these challenges will be overcome in the near future as both academia and industry devote considerable resources in basic and translational research involving NF- $\kappa$ B.

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